Attorney Docket No. 15021-6

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	)	
	)	
Christopher A. Starr and Todd Zankel	)	
	)	Group No.: **1657**
Serial No. **10/501,028**	)	
	)	Examiner: **Kailash C. Srivastava**
	)	
I.A. Filing Date: **January 10, 2003**	)	
	)	
For: **Use of p97 as an Enzyme Delivery Of Therapeutic Lysosomal Enzymes**	)	

The Commissioner of Patents
& Trademarks
Washington, D.C. 20231
U.S.A.

Dear Sir:

DECLARATION UNDER 37 CFR §1.132

I, Wilfred A. Jefferies, a citizen of Canada, and resident of South Surrey, British Columbia declare that the following facts are within my knowledge and are true.

1. I reside at 12596 23rd Avenue, South Surrey, British Columbia, Canada.

2. I am currently a Professor at the University of British Columbia in the Michael Smith Laboratory.

3. I have been conducting research in the field of Immunology since 1981. I have authored over 70 publications which have been published in refereed journals including Nature. I am an inventor on 5 issued or pending United States Patents. I have worked with p97/melanotransferrin for over 18 years and have

patents related thereto. My curriculum vitae is attached to this Declaration as Exhibit A.

4. I have read U.S. Patent Application Serial No. 10/501,028 filed January 10, 2003 (hereinafter "the Application") and understood the disclosure and the claims of the Application.

5. I have read and understood the office action that issued on the Application on August 17, 2009. In particular, I note the Examiner's objection to the specification as not being enabling for a method to treat a subject having a lysosomal storage disease comprising a method wherein a composition comprising a p97 molecule covalently linked to a protein is actually administered to said subject/patient. I respectfully disagree with the objection.

6. As mentioned in my previous Declaration of April 27, 2009, for the treatment of lysosomal storage diseases, the first step is to refit the lysosomal enzyme, either chemically or genetically, with carrier sequences that specify transport from blood to brain and into the lysosome of cells. There are at least four requirements necessary for a carrier to transport across the blood brain barrier:

a. the carrier moiety must be efficiently transcytosed across the brain capillary endothelium and into the interstitial fluid of the brain. This ability must remain unimpeded within the context of an association with the lysosomal enzyme.

b. as the complex appears in the interstitial fluid, affected brain cells must be able to take it up. Expression of receptors can be quite tissue specific so there is no reason to assume that the same receptors involved in transcytosis will be adequately represented on a majority of cells in the brain.

c. the carrier should specify, or at least not interfere with, the lysosomal routing of the enzyme after uptake.

d. the carrier should not interfere with the action of the enzyme on its natural substrate nor should it significantly reduce the lysosomal half-life of the enzyme.

7. p97 meets all of the above criteria as evidenced below.

8. In order to demonstrate that p97 conjugated to a lysosomal enzyme can be delivered to a lysosome in a cell, experiments have been conducted under my general supervision. Firstly, I refer to my April 27, 2009 Declaration which showed the results of an *in vitro* brain barrier model, which showed the appearance of p97 in basolateral chamber over time after spiking the protein into the apical chamber. This phenomena was found to be temperature sensitive and saturable. P97 did not change the permeability of the barrier to sucrose and p97 was transferred between the chambers intact. Also shown was an *in situ* brain perfusion model that demonstrated that the volume of distribution of p97 into parenchyma was 10 times that of the cerebrovascular space marker inulin. Thus, p97 was shown to be transcytosed across brain capillary endothelial cells. Further shown in my April 27, 2009 Declaration was the generation of p97-iduronidase fusions. In addition, it was shown that both fluorescently-labeled p97 and fluorescently-labeled iduronidase were taken up by brain cells and showed a punctate staining pattern characteristic of lysosomal localization.

9. In response to the April 27, 2009 Declaration, the Examiner notes the absence of evidence that a) said composition is comprised of a linker as claimed instantly, and b) specific pharmacokinetic data from the subjects having been administered said linker mediated p97 molecule covalently linked to a protein, or to β-hexosaminidase A. I disagree with the Examiner for the following reasons.

10. Firstly, a linker may or may not be present in the claimed p97 conjugates.

11. Secondly, I attach Exhibit B to this Declaration, which is similar to Exhibit J of my April 27, 2009 Declaration. In particular, Western Blot analysis of various fusions containing linkers in the presence or absence of Cathepsin D is shown using an antibody to either iduronidase (top blot) or p97 (bottom blot). Further, Cathepsin D, which is known to be the predominant lysosomal protease, removed p97 from iduronidase *in vitro* without regard to the linker and p97 was destroyed in this process. Thus, it can be concluded that iduronidase activity against its substrate is unaffected by the fusion with a linker or proteolysis normally present in the lysosome.

11. The Examiner further noted that the results that have been submitted are directed to iduronidase and thus are not enabling for the elected species β-hexosaminidase. In this regard, I attach hereto as Exhibit C a Western Blot of conjugates of p97 and N-acetylglucosaminidase (NAGLU). NAGLU is an example of another lysosomal enzyme conjugated to p97.

12. In addition, the literature contains several examples of conjugates of p97 administered *in vivo*. Attached as Exhibit D is a paper by Yang et al. (Gene Therapy 2007, 14:523-532) entitled "Directing adenovirus across the blood-brain barrier via melanotransferrin (P97) transcytosis pathway in an *in vitro* model". This paper shows that p97 has been successfully conjugated to the extracellular domain of the coxsackie-adenovirus receptor. Further, the paper shows in the accepted blood-brain-barrier model that the conjugate is able to transcytose Ad5 vectors. Ad5 vectors are much larger molecules than the presently claimed lysosomal enzymes. If such a large molecule can be targeted to the brain via conjugation with p97, it would be expected that any lysosomal enzyme would also be successful.

13. Attached as Exhibit E is a paper by Karkan et al. (PLoS ONE, June 2008, Volume 3, Issue 6, e2469, pp. 1-14) entitled "A Unique Carrier for Delivery of Therapeutic Compounds beyond the Blood-Brain Barrier", of which I am a co-

author. This paper demonstrates that p97 conjugated chemically with either adriamycin or paclitaxel, injected intravenously into mice was able to penetrate brain tissue and other organs. Further, it was shown that p97-ADR was able to prolong the survival of animals bearing intracranial gliomas or mammary tumors. The results with paclitaxel show brain localization but the cancers were paclitaxel-resistant so efficacy was not established for that particular conjugate but efficacy was established for adriamycin. Thus, whether p97 is covalently linked or chemically conjugated, it is clear that p97 can be a carrier for proteins or drugs from the blood to the brain. This paper also demonstrates that p97 is able to deliver both DIG and Large Gold (13 nm) particles. Again, if such a large molecule can be targeted to the brain via conjugation with p97, it would be expected that any lysosomal enzyme would also be successful.

14. Attached as Exhibit F is a paper by Moroo et al. (Microcirculation (2003) 10, 457-462) entitled "Identification of a Novel Route of Iron Transcytosis across the Mammalaim Blood-Brain Barrier", of which I am a co-author, which shows that p97 loaded with radioactive iron is also able to deliver iron across the blood-brain barrier when injected into the tail vein of C57Bl/6 mice.

15. The above experiments demonstrate that p97 can be conjugated to different molecules, such as NAGLU, and can be transported across the BBB the established *in vitro* model and *in vivo*. The results from the previous Declaration dated April 27, 2009 and the results found in the application show that p97 can deliver into the lysosome *in vitro*, p97 can be conjugated via a linker to a lysosomal enzyme and p97 can be digested by Cathepsin D releasing the active lysosomal enzyme. These results combined with the known delivery of p97-conjugates to the blood brain barrier and the fact that lysosomal storage diseases require enzyme in the lysosome of affected cells demonstrates that p97 conjugated to lysosomal enzyme would be useful for treating lysosomal storage diseases.

16. In view of all of the above, I believe that the claims as currently on file in the application are sufficiently enabled under 35 USC §112, first paragraph.

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statement and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the Application or patent resulting therefrom.

Nov 12th, 2009
DATE

Wilfred A. Jefferies

Exhibit A

THE UNIVERSITY OF BRITISH COLUMBIA
Curriculum Vitae for Faculty Members

Date: November 10, 2009 Initials: WA

1. **SURNAME:** JEFFERIES **FIRST NAME:** Wilfred
MIDDLE NAME: Arthur

2. **DEPARTMENT/SCHOOL:** Michael Smith Laboratories, Biomedical Research Centre, Medical Genetics, Microbiology & Immunology, and Zoology

3. **FACULTY:** Science, Medicine

4. **PRESENT RANK:** Professor **SINCE:** March 8, 1999

5. **POST-SECONDARY EDUCATION**

University or Institution	Degree	Subject Area	Dates
University of Victoria	B.Sc.	Biochemistry (First Class with Distinction)	1981
University of Oxford	D. Phil. (OXON)	Molecular Immunology	1985

6. **EMPLOYMENT RECORD**

(a) Prior to coming to UBC

University, Company or Organization	Rank or Title	Dates
University of Victoria Department of Biochemistry and Microbiology	Undergraduate Research Assistant	1979
B.C. Cancer Control Agency, University of British Columbia, Terry Fox Lab	Undergraduate Research Project	1980
University of British Columbia Department of Pediatrics	Work-Studies Student	1981
Institut Suisse de Recherches Expérimentales sur le Cancer Lausanne, Suisse	Postdoctoral Research Fellow	1985-1987
Ludwig Institute for Cancer Research, Karolinska Institute, Stockholm, Sweden	Postdoctoral Research Fellow	1987-1989

(b) At UBC

University, Company or Organization	Rank or Title	Dates
University of British Columbia	Assistant Professor	1989-1994
University of British Columbia	Associate Professor	1994-1999
University of British Columbia	Professor	1999-present

(c) **Date of granting of tenure at U.B.C.: July 1, 1994**

7. LEAVES OF ABSENCE

No leaves taken.

8. TEACHING

Session	Course Number	Scheduled Hours	Class Size	Hours Taught			
				Lectures	Tutorials	Labs	Other
1989-90	MICRO 530	36	12	6			
	BIOL 350	36	150	2			
	BIOL 437		2			>100	Research
	BIOL 448		2			"	projects
	BIOL 449		1			"	
1990-91	MICRO 430	36	20	6		>100	Research
	BIOL 437		2				projects
90-91	MICRO 200	72	100	15			
(summer)	BIOL 448		2			"	
	MICRO402/ MED GEN 410	36	40	13 20			
	MICRO 302	36	150	20			
	PHARMAC 521	36	15	1			
1991-92	BIOL 437		2			>100	Research
	BIOL 448		2			"	projects
	MICRO 200	72	275	13			
	MICRO 402/ MED GEN 410 (Course Organizer)	36	100	13			
1992-93	MICRO 200	72	275	13			
	MICRO 530	36	12	6			
	BIOL 437		2			>100	Research
	BIOL 448		2			"	projects
	MICRO 402/ MED GEN 410 (Course Organizer)		100	13			

Session	Course Number	Scheduled Hours	Class Size	Hours Taught			
				Lectures	Tutorials	Labs	Other
1994-95	BIOL 437		2			>100	Research
	BIOL 448		2			"	Projects
	MICRO 200	72	275	13			
	MICRO 402/ MED GEN 410 (Course Organizer)	36	100	13			
1995-96	BIOL 448		3			>100	Research
	MICRO 202	36	275	15	1		Projects
	MICRO 402/ MED GEN 410 (Course Organizer)	36	70	13			
1996-97	BIOL 448		3			>100	Research
	MICRO 202	36	275	15	1		Projects
	MICRO 402/ MED GEN 410 (Course Organizer)	36	70	13			
1997-98	BIOL 448		3			>100	Research
	MICRO 202 -						Projects
	Section 1	36	275	12	1		
	Section 3	36	275	12	1		
	MICRO 402/ MED GEN 410 (Course Organizer)	36	70	13			
1998-99	MICRO 530	36	12	6			
	MICRO 202						
	MICRO 402						
1999-2000	MICRO 202	36					
	MICRO 402	36					
	MED GEN 410	36					
	MICB 430	36					
	MICB 530	36					
2000-2001	MED GEN 410	36	10	16			
		36	10	25			
	MICRO 430	36	10	16			
	MICRO 530						Research
	MICRO 449		2				Projects

Session	Course Number	Scheduled Hours	Class Size	Hours Taught			
				Lectures	Tutorial	Labs	Other
2001-2002	MICRO 530	15	16	5			
	MICRO 430	16	25	4			
	MED GEN 520	16	16	8			
	MICRO 449		2			>100	Research
2002-2003	MICRO 202	15	16	5			
	MICRO 430	15	16	8			
	MICRO 449		1			>100	Research
2003-2004	MICRO 202	15	16	5			
	MICRO 430	15	16	8			
	MICRO 449	1	1			>100	Research
2004-2005	MICRO 402	30	75	26			
	MICRO 449		2			>100	Research
2005-2006	MICRO 402	18	78	12			
	MICRO 449		1			>100	Research
2006-2007	MICRO 402	18	72	12			
	MICRO 449		1			>100	Research
2007-2008	MICRO 402	18	72	12			
2008-2009	MICRO 402	18	76	12			
	MICRO 449		4			>100	Research
2009-2010	MICRO 402	18	78	12			
	MICRO 449		2			>100	Research

1. Graduate Students Supervised

Name of Trainee	Program Type *	Dates		Degree	Year Degree Received	Current position and Institution
		From	To			
Haidl, Ian	PhD -MI	1989	1996	PhD	96	Assistant Professor, Dalhousie NS
Reid, Gregor	PhD -Z	1989	1996	PhD	96	Staff Scientist, Children's Hospital of Philadelphia
Lomas, Cyprien	PhD -Z	1989	1999	PhD	99	Director of the Learning Centre, Land & Food Systems, UBC
Lippe, Roger	PhD -Z	1990	1995	PhD	95	Associate Professor at University of Montreal
Food, Mike	MSc.-MI	1990	1993	M.Sc.	93	Entrepreneur
Yang, Joseph	MSc. -Z	1993	1999	M.Sc.	99	Lawyer with Farris, Vaughn, Wills & Murphy LLP, Vancouver
Alimonti, Judie	PhD -Z	1994	1998	Ph.D.	98	Staff Scientist CDC, Winnipeg
Tiong, Jacqueline	PhD -MI	1994	2001	Ph.D.	01	Director of Corporate Development, Allon Therapeutics, Vancouver
Moise, Alex	PhD -Z	1994	2000	Ph.D.	00	Instructor, Case Western Reserve University, Cleveland
Lizee, Greg	PhD -Z	1993	2000	Ph.D.	00	Assistant Professor, U. Texas, MD Anderson Cancer Center, Houston
Hsu, Forrest	MSc -Z	1995	1997	M.Sc.	97	Medical Doctor (UBC)
Bromm, Michael	MSc-Z	1995	1998	B.Sc.	98	Lawyer, Associate with Lang Michener LLP, Vancouver
Walker, Brandie	PhD -MI	1995	2002	Ph.D.	02	Medical Doctor (U. Calgary)
Kotturi, Maya	PhD -MI	1998	2004	Ph.D.	04	Staff Scientist, La Jolla Institute for Allergy & Immunology, CA
Dickstein, Dara	PhD-MG	1998	2004	Ph.D.	04	Assistant Professor, Mt. Sinai Medical Center, Dept Neuroscience, N.Y.
Grant, Jason	PhD -MI	1999	2005	Ph.D.	05	Research Associate, U. Alberta
Chen, Susan	MSc -Z	2000	2006	—	—	Alternative Medicine Practitioner - Traditional Chinese Medicine
Johnson,Laura	PhD -Z	2000	2004	Ph.D.	05	Assistant Professor, NIH
Biron, Kaan	PhD -MI	2002	Present			Graduate Student, UBC
Tian, Mei Mei	PhD -MI	2002	2009	Ph.D	09	Postdoctoral Fellow, UBC
Wilcox, Sara	PhD -MI	2002	Present			Graduate Student, UBC

Seipp, Robyn	PhD -Z	2002	2008	Ph.D.	08	Postdoctoral Fellow, CDRD Vancouver
Setiadi, A. Francesca	PhD -Z	2002	2007	Ph.D.	07	Post-doctoral Fellow, Stanford CA
Omilusik, Kyla	PhD -MI	2003	Present			Graduate Student, UBC
Young, Joanne	PhD -Z	2003	Present			Graduate Student, UBC
Wang, Teresa	MSc -MI	2005	Present			Graduate Student, UBC (on business training leave)
Garduno, Alonso	MSc -MI	2005	2007	—	—	Medical School, Mexico City
Hartikainen, Jennifer	PhD -MI	2006	Present			Graduate Student, UBC (on personal leave)
Saranchova, Iryna	MSc -MI	2009	Present			Graduate Student, UBC
Murphy, Elizabeth	MSc -Z	2009	Present			Graduate Student, UBC
Chavez Steenbock, Ana	MSc -Z	2009	Present			Graduate Student, UBC

* Z = Zoology
MI = Microbiology & Immunology
MG = Medical Genetics

Post-doctoral Fellows Supervised

1991-1992 Jonas Ekstrand (Swedish Research Council Fellowship)
1991-1992 Sylvia Rothenberger (Swiss National Research Foundation)
1994-1996 Catherine Barbey (Swiss National Research Foundation)
1995-1997 Iku Moroo (Chiba University Graduate School of Medicine)
1999-2003 Cheryl Pfeifer (MRC/CIHR Operating Grant)
1999-2004 Maki Ujiie (Human Frontier Program Post-Doctoral Fellowship)
2004-2006 Ya Ke (Hong Kong Polytechnic Fellowship)
2004-2008 Anna Reinicke (CIHR Fellowship in Transplantation)
2009-2009 M. Arshad Chaudhry (Contract Research Agreement - biOasis)
2009-present Mei Mei Tian (Contract Research Agreement - biOasis)
2009-present Farnoosh Tayyari (Contract Research Agreement - biOasis)
2009-present Youngmei Gong (Contract Research Agreement - biOasis)

Research Associates

1991-1998 Reinhard Gabathuler (Tobacco Research Council)
1993-1994 Chris Nicol (Synapse Technologies)
1995-1998 Malcolm Kennard (Synapse Technologies)
1999-2003 Qian-Jin Zhang (TapImmune Pharmaceutical CRA)
1999-2007 Genc Basha (MRC/CIHR Operating Grant)
2001-2005 Yuanmei Lou (TapImmune Pharmaceutical CRA)
2002-2006 Tim Vitalis (TapImmune Pharmaceutical CRA; CIHR Operating Grant)

2004-present	Cheryl Pfeifer (UBC)
2009-present	Tim Vitalis (Contract Research Agreement - biOasis)

• Continuing Education Activities

•Host Visiting Scientists (indicate university/organization and dates)

1. Simon Hunt, PhD, Sabbatical Visitor (University of Oxford, U.K.), 1996
2. Iku Moroo, Chiba University School of Medicine, Chiba, Japan, 1997-98
3. Simon Hunt, PhD, Visiting Scientist (University of Oxford, U.K.), 2007
4. Stephen McQuaid, Visiting Scientist (Queen's University, Belfast, Ireland), 2008

Other

Thesis Committee member for: (26)	**Supervisor**
Bruce Banfield	Frank Tufaro
Peter Cheung	Frank Tufaro
Kathy Horley	Fumio Take
Andrew Pyszniuk	Fumio Takei
Clay Welder	Don Moerman
Andrea Ingram	Don Moerman
Carmine Carpenito	Don Moerman
Mary Gilbert	Don Moerman
Richard Hegele	Jim Hogg
William Craig	Connie Eaves/Peter Lansdorp
Stephen Land	Peter Hochachka
David Hunt	Julia Levy
Chris Fraser	Keith Humphries
Patrick Rebstein	Gerry Weeks
Arpeta Matia	Pauline Johnson
Bevan Voth	Rob McMaster
Jaime Bellatin	Rob McMaster
Amanda Jones	Hermann Ziltener
Jose Rey-Ladino	Niel Reiner
John Chiu	Alan Eaves
Madelaine Lemieux	Connie Eaves
Kelly Brown	Pauline Johnson
Julia Boyle	Hung-Sia Teh
Jennifer Cross	Pauline Johnson
Mark Bleakley	Ross MacGillivray
Ann Wong	Ross MacGillivray

Dissertation Committee member for: (19)	
Chris Fraser, Ph.D.	Keith Humphries
Mark Daly, MSc.	Hugh Brock
Carmine Carpenito, MSc	Fumio Takei
Peter Cheung, MSc.	Frank Tufaro
Mike Food, M.Sc.	Wilfred Jefferies
Clay Welder, M.Sc.	Fumio Takei

Richard Hegele, Ph. D.	Jim Hogg
William Craig, M.Sc.	Connie Eaves/Peter Lansdorp
Helena Chaye, Ph.D.	Shirley Gillam
Brad Spiller, Ph. D.	Dana Devine
Mary MacDonald Ph.D.	W. Rob McMaster
Charlotte Morrison Ph.D.	W. Rob McMaster
Andrew Pyszniuk Ph.D.	Fumio Takei
Arpita Maiti, Ph.D.	Pauline Johnson
Angus Murray, Ph.D.	W. Rob McMaster
HB Choi, Ph.D.	James McLarnon
Jae Kyu Ryu, Ph.D.	James McLarnon
Klaus Gossen, Ph.D.	Hermann Ziltener
David Hudson, Ph.D.	Ross MacGillivray
External examiner: (2)	
Mark Luscher Ph. D. Toronto	Brian Barber
Jay C. Varghese Ph.D. U. Alberta	Kevin Kane

2. SCHOLARLY AND PROFESSIONAL ACTIVITIES

(a) Areas of special interest and accomplishments

I have a longstanding interest in the mechanisms of induction of immune responses. According to the "Faculty of 1000", I have authored several widely read and downloaded papers in the areas of Immunology, Cell Physiology and Pathology. These include the identification of the process whereby dendritic cells trigger primary immune responses against foreign pathogens and cancer cells. This study, published in Nature Immunology, was amongst the most downloaded research papers for all of Biology in 2003 and it remains a highly cited finding in Immunology that has revised the pages of many Immunology texts. In addition, our study, published in PLoS Pathogen in 2005, identified a new method for enhancing and extending the vaccine supply against many foreign pathogens. These studies appear to provide a solution to a tremendous societal problem of meeting the needs for vaccine supplies in the face of emerging pandemics.

Area 1: Antigen presentation to naïve T cells and cross-priming by dendritic cells (DCs) is the key event in stimulating most, if not all adaptive immune responses against foreign antigens. Though critically important in harnessing the power of the immune system to eradicate disease, the cross priming pathways in DCs are not yet identified. For many years, immunologists have hypothesized the existence of multiple cross-priming pathways and our group was the first to identify a pathway leading from the DC cell surface into an endolysosomal peptide loading compartment. During this period, others have worked on various other models of cross priming involving the highly controversial Ergosome processing model. Parallel with these studies is a nearly universal observation that there exists in DCs, a so-called TAP-independent cross-priming pathway. There are literally 100s of papers that suggests this latter pathway exists but no one has been able to discover this pathway. Our new study, submitted to ***Science*** for publication, describes this pathway for the first time. Our work began by asking the fundamental question whether the invariant chain (Ii, Cd74), a protein chaperone of MHC Class II proteins, can play a key role in cross-presentation and cross-priming by DCs of cytolytic T cells in the context of MHC I molecules. Specifically, we addressed whether Ii delivers a subset of MHC Class I proteins from the endoplasmic reticulum through to the endolysosomal compartments of DCs, where

exogenous antigens are acquired for subsequent cross-presentation and priming CTL cell responses. We demonstrate that this new pathway is involved in immune response to many types of physiological antigens and we show this pathway is absolutely required for immune responses *in vivo* as the loss of this pathway abolishes immune responses. Our principal discoveries are that MHC Class I molecules interact with Ii protein in DCs and following this interaction in the endoplasmic reticulum, the complex egresses to the endolysosome. Furthermore, we demonstrate that MHC I/Ii complex formation and subsequent MHC I peptide loading in the endolysosomal compartment is TAP-independent as it still occurs in DCs from TAP-deficient animals. We then used Ii deficient mice to provide definitive evidence that the Ii functions normally in mediating MHC I cross-priming in many types of infection. This new discovery has direct implications for the clinical community, particularly in the design of targeting vaccine candidates to the Ii-dependent MHC I cross priming compartment for stimulating protective immunity against infectious diseases and potentially for learning how to reduce tissue and stem cell rejection between heterologous donors and recipients.

Area 2: I am a leader in the identification a molecular mechanism by which cancer cells evade destruction by the host immune response. These finding are significant because they show that the antigen processing defect in these carcinomas reduces tumour recognition and they highlight the potential of TAP(s) to augment immune responses in an MHC unrestricted manner, thereby alleviating the need to identify the exact tumour antigen from each individual tumour. These study point the way toward a new method for immune therapy against many types of metastatic tumours. This was a significant discovery that still reverberates as a significant finding in the field. It has spawned no less than 200 research papers from other cancer researchers. I have used these findings to develop completely novel immunological approaches to eradicate tumors. These approaches are a departure from other therapeutic modalities because they provide a bridge between the fields of vaccines and gene therapy, and provide a generalized immunotherapeutic approach for cancers of various types. In addition, these findings have also provided prognostic indicators of cancer progression and have been widely hailed to be of fundamental importance for understanding and treating cancers of many kinds. This translational research now forms a basis for generalized cancer therapy that has moved from the bench forward into clinical trials. We have now extended these studies into the area of epigenetic regulation of antigen process gen programs in Cancer cells. The histone deacetylase inhibitors (HDACi) have been hailed as a powerful new class of anti-cancer drugs. The HDACi, trichostatin A (TSA), is thought to interfere with epigenetic control of cell cycle progression in G1 and G2-M phase, resulting in growth arrest, differentiation, or apoptosis. We have now described a novel mechanism of action of HDACi's in promoting immune responses against tumors. We recently report that treatment of carcinoma cells with TSA increases the expression of many components of the antigen processing machinery, including TAP-1, TAP-2, LMP-2 and Tapasin. Consistent with this result, we found that treatment of metastatic carcinoma cells with TSA also results in an increase in MHC class I expression on the cell surface that functionally translates into an enhanced susceptibility to killing by antigen-specific CTLs. Finally, we observed that TSA treatment suppresses tumor growth and increases TAP-1 promoter activity in TAP-deficient tumor cells *in vivo*. Unexpectedly, this *in vivo* anti-tumoral effect of TSA is entirely mediated by an increase in immunogenicity of the tumor cells, as it does not occur in immunodeficient mice. These novel insights on epigenetic control mechanisms regulating tumor immune escape may help revise immunotherapeutic modalities for eradicating cancers.

Area 3: Similar to the immuno-evasion mechanism described in tumours, we have described a unique viral immunosubversion mechanism, involving a small adenoviral protein that mimics a host cell protein, thereby tricking the cell into postponing apoptosis and facilitating viral propagation. The ability of adenoviruses to subvert this apoptosis pathway has been known for many years, yet the E3-6.7K

protein we described has never been linked to this pathway, and indeed has long been thought to be too small to have significant effect. This is an important finding since viral infection of cells normally triggers cell death pathways and viral mechanism for subverting the host must be understood in order to overcome and treat pathogens.

Area 4: The transport of calcium ions (Ca^{2+}) is essential for immunoreceptor signaling, including lymphocyte antigen receptors and granulocyte Fc receptors, and plays important roles in the regulation of lymphoid quiescence, immune cell function and differentiation. Rises in intracellular Ca^{2+} concentrations are mediated through two interconnected and complementary mechanisms: the release of endoplasmic reticulum Ca^{2+} "stores" and "store-operated" calcium entry via plasma membrane Ca^{2+} channels. However, the identity of the molecular components that regulate Ca^{2+}-oscillations that participate in thymocyte selection and T cell homeostasis are unclear. We have published a series of important papers that demonstrate that the pore-forming subunit of a Ca^{2+} channel, $Ca_V1.4$ is intrinsic in the development and the survival of naïve CD4 and CD8 T cells. This isoform of $Ca_V1.4$ is critically required for the regulation of intracellular Ca^{2+} stores and modulates TCR-induced calcium fluxes, impacting Ras and ERK activation and IL-7 responsiveness. Collectively, these studies have revealed hitherto unrecognized functions of $Ca_V1.4$ in balancing naïve T cell homeostasis and generating functional T cell immune responses. Other researchers have long pursued this but we were the first to clone and identify the molecular features of the channel that appears to be the master switch in controlling T cell responses. As such it is also a key target for identifying new classes of immune therapeutics. The latest paper in these studies is submitted to the journal, ***Immunity***.

Area 5: Earlier in my career as a graduate student at Oxford University, I was also interested in the role of iron in promoting cancer cell growth and was the first to define a pathway of iron acquisition by melanomas. These discoveries lead directly to methods of removing cancer cells from autologous bone marrow: a technology that is still in use today. Furthermore, I was instrumental in defining the receptor system for the passage of iron from the blood into the brain. This discovery has lead to the development of new drug delivery systems for the delivery of anti-cancer drugs into the brain for the treatment of brain tumors. As therapeutic intervention in many neurological diseases is thwarted by the physical obstacle formed by the blood-brain barrier (BBB) that excludes most drugs from entering the brain from the blood, identifying efficacious modes of drug delivery to the brain remains a "holy grail' in molecular medicine and nanobiotechnology. Our studies provide the initial proof of concept for any protein-based carrier capable of shuttling therapeutic levels of drugs from the blood to the brain for the treatment of neurological disorders, including classes of resident and metastatic brain tumors. These observations have been the impetus for the creation of an entirely new area of drug development used for the treatment of other diseases of the brain, including lysosomal storage diseases such as Tay-Sachs and Sandhoff disease. These studies are widely cited and have had an immense impact on understanding the biology of the blood brain barrier.

Area 6: My continued interest in iron transport to the brain, led to making the discovery of the first unique marker of reactive microglia associated with the senile plaques in Alzheimer's disease brains. This remains the first and only marker of its kind. We subsequently demonstrated that the marker molecule, called p97, is elevated in the blood and cerebral spinal fluid of individuals with Alzheimer's disease. Clinical trials now support the hypothesis that this molecule is the first stand-alone serum protein marker for this disease and these findings may form the basis of a test to screen for Alzheimer's disease and also to monitor the success of emerging therapeutics for the treatment of Alzheimer's disease.

Area 7: As a result of my interest in the Blood-Brain Barrier, my group recently demonstrated that this barrier is impaired in Alzheimer's disease prior to disease onset and the appearance of plaques in the brain. These pioneering studies have again created the seed for another new area of scientific investigation that is being actively followed up by the research community. I was awarded the Wiederhelm Award prize for this research. Furthermore, these finding link Alzheimer's disease and stroke in a unique and fundamental way and may form the basis to understand modality of AD vaccines and other methods for treating this disease.

Quality of publications: Many of my scientific papers are published in the world's most outstanding scientific journals including ***Nature, Proceedings of the National Academy of Science, Nature Immunology, Public Library of Science One, Journal of Experimental Medicine, EMBO Journal, Nature Medicine, FASEB Journal, Journal of Biological Chemistry, Nature Biotechnology, Public Library of Science Pathogens and in many Cancer and Immunology and Neurobiology related journals, including the Cancer Research, the Journal of Immunology*** and ***Brain Research***. In addition, I have published several reviews in the prestigious Trends Journals and I am the author of innumerable conference proceedings.

Presentations: I am internationally recognized as a scientific leader and innovator, and continue to present our work as an invited speaker to international meetings including Gordon Conferences and Keystone Meetings as well as many international congresses. My research has impacted the scientific awareness of the general public through third party commentaries in the popular press. These include popular journals such as ***Readers Digest, Newsweek, Scientific American, Popular Mechanic*** and newspapers such as the ***New York Times***, the ***Daily Mail***, the ***Washington Post*** and the ***Globe and Mail***, just to name a few.

Record of grant funding: I have a strong record of peer reviewed grant funding. I currently hold five CIHR grants, a Western Economic Diversification grant, and a Multiple Sclerosis Research grant. Other grants I have held include the Sanfilippo Chilldren's Research Foundation, Genome Canada, Prostate Cancer Research Foundation of Canada, and grants from the National Institute of Health in the USA, the Natural Science and Engineering Council of Canada, the Canadian Network of excellence for vaccine Development, the National Cancer Institute of Canada and the Canadian Foundation for AIDS Research.

Mentorship: I have an extensive record as a mentor of graduate students. I have supervised 28 Doctoral students, 20 of which have graduated so far during my tenure at UBC. Many of these students now hold independent professorships at Universities located throughout the globe. I have mentored over 50 undergraduate research projects in my lab and have taught multiple courses at every level of undergraduate education.

Impact of infrastructure at University: As Director of the UBC Life Sciences Centre Transgenic Facility, I participated in the writing and presenting the University of British Columbia's Canadian Foundation for Innovation (CFI) award entitled "Centre for Disease Modeling", for which we were awarded an aggregate of 80 Million dollars. The CFI award will allow the creation of extensive infrastructure for the UBC Life Sciences Centre Animal Care facility. I was a co-investigator on the successful Genome Canada award, "North American Conditional Mouse Mutagenesis Project", with total funding of over $8 million between three Genome Centres across Canada. This Genome Canada Award provides operating funds for state-of-the-art research at UBC. In addition, I have been a driving force in developing novel transgenic techniques and services that should allow the investigation of new models of human diseases such as cancer. I serve as the Director of three separate animal facilities at

the University of British Columbia, including: the Small Mammal Unit at Zoology South Campus, the University of British Columbia Transgenic Animal Facility and the University of British Columbia Rederivation Facility. The latter two facilities have provided state of the art animal technologies to the greater biotechnology and university community. I have spent literally hundreds of hours, directing, organizing and writing grants to fund these animal facilities at the University of British Columbia for the entire University community. I am also the Director of the Rederivation Facility at the University of British Columbia and negotiated with the Federal Government of Canada for the funding of a new multi-million dollar Animal Rederivation Facility building on campus that is now completed and providing services. I have also been involved in leading a new group called TransArc Network that is a collaboration between the University of British Columbia, the University of Victoria, the University of Northern British Columbia, Simon Fraser University, the British Columbia Cancer Control Agency, the Terry Fox Cancer Research Centre, the Jack Bell Research Centre, the Centre for Molecular Medicine and Therapeutics coordinate all transgenic research animal services across the province. These Units are now operated under funding I received from the Federal Government of Canada's Western Diversification Fund for a new Pan-British Columbia Animal Research Services Network for which I provided the vision. This is the first of its kind in the world where mutlicentre animal facilities will unite the services they provide individually into a single point of entry animal service provider. This Network is now established and is providing transgenic and toxicology animal services to the whole community within the province of British Columbia and we plan to expand the user base to include all of North America. Furthermore, this network will grow by added additional capabilities such as high containment facilities for dangerous pathogens that will expand research capacity for researchers across North America.

Recognition: I have received recognition as the recipient of a University Killam Faculty Research Fellowship and has received numerous other fellowships and scholarships throughout my career. I have also been awarded the Microcirculatory Society's Wiederhelm Award in 2008.

Commercialization and out-licensing: I hold no less than 20 issued patents that cover many of my discovered. These international patents form the core technology foundations of four biotechnology and pharmaceutical companies that have employed dozens of individuals over the past 15 years related to the development of my discoveries.

(b) Research or equivalent grants (indicate under COMP whether grants were obtained competitively (C) or non-competitively (NC))

Granting Agency	Subject	COMP	$ per year	Year	Principal Investigator	Co-Investi-gator(s)
National Cancer Institute of Canada	Molecular studies on the transport, recognition and inhibition of MHC Class I gene products	C	153,627	89-94	Jefferies	
National Cancer Institute of Canada	**Equipment** for above	C	36,610	89-91	Jefferies	
National Cancer Institute of Canada	Molecular studies on the transport, recognition and inhibition of MHC Class I	C	183,708	91-94	Jefferies	

	gene products and characterization of the melanoma-associated protein, melanotransferrin					
National Cancer Institute of Canada	Viral and genetic mechanisms which lower MHC Class I cell surface expression	C	103,000	94-97	Jefferies	
British Columbia Health Research Foundation	The role of the E3/19k protein of Adenovirus-2 in lowering the cell surface expression of HLA molecules and in viral persistence	C	45,000 50,000	89-90 90-91	Jefferies	
British Columbia Health Research Foundation	The function of the melanoma associated protein melanotransferrin	C	27,000	92-94	Jefferies	
British Columbia Health Research Foundation	**Equipment** for Molecular action of cyclosporin A	C	14,000	89-90	Jefferies	
British Columbia Health Research Foundation Emergency	**Equipment** – Cryopreservation unit	C	5,000	91-92	Jefferies	
Natural Science and Engineering Council of Canada	**Equipment** for The generation of antigen presenting mutants	C	25,000	1990	Jefferies	
Natural Science and Engineering Council of Canada	**Equipment** – Cryopreservation unit	C	8,000	90-91	Jefferies	
American Council for Tobacco Research	The role of the E3/19k protein of Adenovirus-2 in lowering the cell surface expression of HLA molecules and in viral persistence	C	543,400	91-96	Jefferies	
Vancouver Foundation/Medical Services Association	An early detection test for Alzheimer's disease	C	45,000	91-93	Jefferies	
Medical Research Council of Canada	Human melanotransferrin	C	74,000	93-97	Jefferies	
Medical Research Council of Canada	Molecular characterization of antigen processing variants	C	60,000	93-97	Jefferies	
B.C. Science Council	An early detection test for Alzheimer's disease	C	149,000	95-98	Jefferies	

National Centre of Excellence for Neural Regeneration	Transport across the Blood Brain Barrier	C	48,000	93-98	Jefferies	
Medical Research Council of Canada	Characterisation of the function of Melanotransferrin	C	52,673	97-00	Jefferies	
Medical Research Council of Canada	Antigen Processing in the Context of MHC Class I molecules	C	94,359	97-00	Jefferies	
Natural Science and Engineering Research Council of Canada	Novel eukaryotic expression Systems	C	108,760	97-00	Jefferies	
National Cancer Institute of Canada	Animal models of adenovirus persistence	C	25,356	97-98	Jefferies	
National Cancer Institute of Canada	Studies on the structure and function of MHC Class I molecules	C	25,356	97-98	Jefferies	
National Cancer Institute of Canada	Studies on exogenous pathway of antigen presentation	C	111,000	99-02	Jefferies	
NSERC	Novel recombinant protein production system based on expressing GPI-anchored proteins in Dictyostelium discoideum	C	108,760	97-00	Jefferies	
CANVAC	Enhancing efficiency of antigen presentation using TAP based immunotherapy	C	28,050	00-02	Jefferies	
Canadian Institute of Health Research	Studies on antigen processing in the context of MHC class I molecules	C	126,028	00-04	Jefferies	
National Cancer Institute of Canada	Maintenance of calcium ion homeostasis , a new viral immuno-evasion mechanism	C	118,990	01-04	Jefferies	
BCRP Concept Award: U.S. Army Medical Research Acquisition Activity	Novel Immunotherapy for Malignant Breast Carcinomas		50,000 US	01-02	Jefferies	
CANFAR	Identification of cellular interaction of MHC I cytoplasmic domain displayed by HIV nef.	C	80,000	01-02	Jefferies	
Canadian Institute	The role of p97 and a novel	C	124,065	01-	Jefferies	

of Health Research	transferrin receptor homologue in metal uptake			06		
Canadian Institute of Health Research	Delivery of therapeutic proteins across the blood-brain barrier	C	59,000	04-06	Jefferies	
Canadian Institute of Health Research	Studies on antigen processing in the context of MHC class I molecules	C	131,070	04-08	Jefferies	
Canadian Institute of Health Research	Aspects of antigen presentation by breast and lung carcinomas and dendritic cells	C	104,133	05-08	Jefferies	
Western Economic Diversification	Support for the Rederivation Facility	C	900,000	04-08	Jefferies	
Canadian Foundation for Innovation	UBC Centre for Disease Modeling	C	125,117	04-09	Teh	Jefferies, Hancock, Jean, Brunham, Finlay, Lefebvre, McNagny, Brooks,Kieffer
Canadian Institute of Health Research	Research Resource Equipment Grant - Determinants of genome stability	C	$125,325	05	Phil Hieter	Finlay, Foster, **Jefferies**, Kronstad, Measday, Snutch
Sanfilippo Children's Research Foundation	Development of a therapeutic tool for the delivery of proteins across the blood-brain barrier	C	$80,000	06-08	"	
Genome Canada	NorCOMM: North American Conditional Mouse Mutagenesis Project	C	2,000,000	06-07	Geoff Hicks/Janet Roussant	**Jefferies**, Stanford, Wurst, Bradley, Skarnes, Ding, Rancourt, Nagy, Hughes, Hoodless, Marra, Roder, Gondo, Lefebvre, MacVicar, Hannon, McKerlie, Lloyd, Einsiedel
Canadian Prostate Cancer Research Foundation	Regulators of prostate cancer immunogenicity	C	$60,000	06-07	Jefferies	
Michael Smith Foundation for Health Research	BC Mouse Models Platform - Technology/Methodology Platform Award for LOI	C	$50,000	06	Jefferies	Payne, Leavitt

Multiple Sclerosis Society of Canada	Pilot Project Award	C	$35,000	08	Jefferies	McQuaid
Canadian Institute of Health Research	Studies on antigen presentation in dendritic cells	C	$140,000	08-13	Jefferies	
Western Economic Diversification	BC Pre-Clinical Research Consortium	C	1,500,000	08-10	Jefferies	Harvey-Clark, Bally
Canadian Prostate Cancer Research Foundation	Regulation of antigen processing machinery in prostate carcinomas	C	$60,000	08-09	Jefferies	
Canadian Institute of Health Research	Dissecting the molecular mechanism of HIV nef immunosubversion	C	100,000	08-09	Jefferies	
Canadian Institute of Health Research	Studies on p97 (Melanotransferrin)	C	100,000	09-10	Jefferies	
Canadian Stroke Network/ CIHR	Vascular Pathology in Models of Alzheimer's Disease	C	100,000	09-10	Jefferies	
Canadian Institute of Health Research	Regulation of antigen processing machinery in carcinomas	C	480,608	09-13	Jefferies	
Canadian Institute of Health Research	First annual symposium for the BC-Preclinical Research Consortium	C	15,000	09-10	Jefferies	Harvey-Clark

(c) Research or equivalent contracts (indicate under COMP whether grants were obtained competitively © or non-competitively (NC).

Contract Agency	Subject	COMP	$ per year	Year	Principal Investigator	Co-Investigator(s)
Biomarin/Synapse Technologies Inc	AD diagnostics	C	$125,000	96-02	Jefferies	
TapImmune Inc	Novel immunotherapy for malignant carcinomas	N	$125,000	00-05	Jefferies	
TapImmune Inc	TAP Vaccines	N	$147,348	05-06	Jefferies	
INEX	To determine biodistribution of siRNA using fluorescent microscopy techniques	N	$24,000	07	Jefferies	
biOasis	P97 as a biomarker for Alzheimer Disease	N	190,375	09-10	Jefferies	
biOasis	P97 blood-brain barrier project	N	248,375	09-10	Jefferies	

(d) Coordinated University Grants:

Title of Grant: BC Pre-Clinical Research Consortium
Funding Source and Program Name: Western Economic Diversification Canada
Amount and Duration: $1,500,000 over 3 years (2008 - 2010)

Title of Grant: Support for the Rederivation Facility
Funding Source and Program Name: Western Economic Diversification Canada
Amount and Duration: $900,000 over 5 years (2004 - 2008)

Title of Grant: University of British Columbia Centre for Disease Modeling
Funding Source and Program Name: Canada Foundation for Innovation (CFI)
Amount and Duration: $22,00,000 over 6 years (2004 - 2009) Aggregate $80 Million dollars

(e) Invited Presentations

1. 1997 Molecular Biology of Alzheimer's Disease, IBC Conference San Francisco, California, p97 and Alzheimer's Disease
2. 1998 Round Table discussion on Biomarkers of Alzheimer's Disease International Congress of Alzheimer's and related diseases, Amsterdam
3. 1998 Toward a Comprehensive Theory for Alzheimer's Disease Orlando Florida, AD Association
4. 2002 Gordon Conference, Barriers of the Central Nervous System. New Hampshire, USA.
5. 2002 Annual Canadian Vaccine Meeting. Toronto, Ont., Canada.
6. 2002 Progress in Vaccination against Cancer (PIVAC). British Society for Immunology. Nottingham, England.
7. 2003 Keystone Conference – Tumor Immunology Keystone, Colorado
8. 2005 Keystone Conference – Tumor Immunology II. Keystone, CO
9. 2006 9th International Symposium on Dendritic Cell - Edinburgh, Scotland (delivered by graduate student Robyn Seipp)
10. 2006 Vaccines on the Horizon - 7th Canadian Immunization Conference, Winnipeg, MB
11. 2007 Prostate Cancer Research Retreat, Canadian Prostate Cancer Research Foundation, Oakley, ON (delivered by graduate student Francesca Setiadi).
12. 2007 Vaccine Evaluation Centre, Vancouver, BC April 2007
13. 2007 Keystone Conference – Cancer Vaccines. Banff, AB March/April 2007
14. 2007 Cerebral Vascular Biology Conference, Ottawa, ON June 2007
15. 2007 Gordon Conference, Antigen Cross-Presentation, Big Sky Montana, Sept 2-7, 2007
16. 2008 University of British Columbia, Establishment of TransARC Network, Vancouver, BC Sept.
17. 2009 Canadian Society of Immunology, 22nd Annual Meeting & 2nd Cancer Immune Therapy Symposium, Whistler, BC " Tumor Immune Therapy: Trials and Tribulations ", April 3-6, 2009
18. 2009 Infection, Inflammation and Immunity (I3) at Life Sciences Institute, UBC, Vancouver, BC. "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours" April 2009
19. 2009 BC Cancer Agency: "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours", Vancouver, BC, June 5, 2009.
20. 2009 University of Insubria, Varese, Italy "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours", June 23, 2009
21. 2009 Ludwig Institute, Lausanne, Switzerland "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours", June 26, 2009

22. 2009 Karolinska Institute, Stockholm, Sweden "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours", July 3, 2009
23. 2009, Sir William Dunn School of Pathology Oxford, "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours",July 9, 2009
24. 2009 University of Montreal. "Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumours", August 14, 2009

(f) Other Presentations

1. 1996-1997 Newspaper interviews
2. Outreach presentations to elementary schools – "Jurassic School"
3. CBC Radio interview
4. 2002 Popular Mechanics
5. 2002 Vancouver Courier
6. 2002 BTV, Business Television
7. 2003, Vancouver Sun
8. 2003, Ubyssey
9. 2004, Vancouver Sun
10. 2004, CBC Radio
11. 2005, BC Cancer Agency, Victoria BC
12. 2006, SHI Consulting interview for NorCOMM
13. BioSpace Beat: TapImmune Corp. TAP-1 Technology Corroborated In Models Of Skin Cancer http://www.biospace.com/news_story.aspx?StoryID=16079120&full=1
14. Vancouver Sun: Canwest News Service, byline by Pamela Fayerman: "Less vaccine needed: Study", Jan 26, 2006
15. Vancouver Sun: Front Page: Canwest News Service, byline by Pamela Fayerman: "UBC researchers make pandemic flu vaccines safer, more effective: Vaccine effective at 1% of normal dose after immune-system booster added", Jan 26, 2006
16. Victoria Times Colonist: Canwest News Service, byline by Pamela Fayerman: "Researchers harness genes to create new vaccines", Jan 26, 2006
17. Montreal Gazette: Canwest News Service, byline by Pamela Fayerman: "Profs give boost to vaccines: An added immune system gene makes lower doses more effective, study finds", Jan 26, 2006
18. Edmonton Journal: Canwest News Service, byline by Pamela Fayerman: "Low-dose vaccines work in tests: Stockpiles would last longer in pandemic", Jan 26, 2006
19. Calgary Herald: Canwest News Service, byline by Pamela Fayerman: "Scientists develop low-dose vaccines", Jan 26, 2006
20. http://strategis.ic.gc.ca/epic/site/p-pp.nsf/en/ph01656e.html "Diagnostics and Therapeutics Update Publication that Pinpoints TAP Defect in Metastatic Carcinomas" August 25, 2005
21. UBC Reports, Dec 4, 2004, Vol. 49, No. 12 "In the News" Compiles by Brian Lin.
22. Calgary Herald: Canwest News Service, byline by Chad Skelton: "Alzheimer's find may allow early treatment: Warning sign discovered in mice", March 4, 2004
23. Edmonton Journal: Canwest News Service, byline by Chad Skelton: "Early sign of Alzheimer's reported: Patients could be treated decades sooner, UBC researchers say", March 4, 2004
24. Victoria Times Colonist: Canwest News Service, byline by Chad Skelton: "Research points to early warning of Alzheimer's", March 4, 2004

25. Vancouver Sun newspaper: Front Page: Canwest News Service, byline by Chad Skelton: "Signs of disease could start in early 20s: UBC discovery creates hope for early Alzheimer's treatment", March 4, 2004
26. Northern Daily News (Kirkland Lake): Canwest News Service, byline by Chad Skelton: "Alzheimer's warning may soon be reality", March 4, 2004
27. 2006 Weather Channel program on vaccines breakthrough
28. CIHR Highlights of Vaccine Study Impact 2005
29. Prostate Cancer Research Association News 2006
30. NATURE Medicine, 2006, Vol. 12, No. 2, p 173. Research Highlights: "Transport genes speed vaccines".
31. UK Daily Mail, November 2007: Cancer cells hide from immune system with invisibility cloak
32. ScienceDaily: Nov 10, 2007: 'Instruction Manual' That Tells Cancers How To Hide From Immune System Discovered (www.sciencedaily.com)

(g) Conference Participation

Organizer:

2nd Annual Cancer Immune Therapy Symposium, Whistler, BC, April 6, 2009, which was held in conjunction with the Canadian Society of Immunology, 22nd Annual Meeting.

Conference Seminar Presenter:

1. ***Jefferies, W.A.,*** Characterization of the rat transferrin receptor. Harden Conference on the Cell Surface Proteins of Lymphocytes, Kent, England, 1982.
2. ***Jefferies, W.A.***, A monoclonal antibody against rat transferrin receptor does not detectably label lymphopoietic stem cells. EMBO Workshop on T Lymphocyte Cloning, Marseille, France, 1983.
3. ***Jefferies, W.A.***, Structure of the CD4(W3/25) T-Helper Lymphocyte Glycoprotein, Biochemical Society, London, England, 1985.
4. ***Jefferies, W.A.***, Structure of the rat CD4(W3/25) glycoprotein of T helper lymphocytes. Sixth International Congress of Immunology, Toronto, Ontario, Canada, 1986.
5. ***Jefferies, W.A.***, Using antisense segments of the H-2 K^k to inhibit cell surface expression of MHC Class I proteins. H-2 and HLA Workshop, Montreaux, Switzerland, 1986.
6. ***Jefferies, W.A.,*** The E3/19K protein of Adenovirus 2 binds to HLA molecules intracellular and inhibits recognition by cytolytic T lymphocytes, International Congress of Virology, Edmonton, Alberta, Canada, 1987.
7. ***Jefferies, W.A.***, A Null MHC Class I Restriction Element Becomes Functions in Transgenic Mice, H-2 and HLA Workshop, Airlie House, Virginia, U.S.A., 1988.
8. ***Jefferies, W.A.***, The Joint Swedish-Israeli Workshop on Biophysical Interactions, Stockholm, Sweden -Studies on MHC Class I molecules, 1988.
9. ***Jefferies, W.A.***, Using antisense RNA in the inhibition of H-2 molecules. EMBO/INSERM Workshop on Antisense RNA, Savoie, France, 1988.
10. ***Jefferies, W.A.***, Cytolytic T cells recognize a chimeric MHC Class I antigen expressed in influenza A infected transgenic mice. The Swedish-Israeli Workshop on the molecular basis of biological recognition, membrane dynamics and transport. Sodergarn Mansion, Stockholm, 1988.
11. ***Jefferies, W.A.***, A murine cell variant differentially presents antigens derived after VSV or Influenza infection. Taos, New Mexico, Keystone Symposium, 1992.
12. ***Jefferies, W.A.***, The adenovirus E3/19K protein blocks the phosphorylation of MHC molecules. Taos, New Mexico, Keystone Symposium, 1992.

13. ***Jefferies, W.A.,*** Differentiated processing of viral and allogeneic peptides. Taos, New Mexico, Keystone Symposium, 1992.
14. ***Jefferies, W.A.,*** Phosphorylation of MHC takes place in a Post-ER compartment, Canadian Society of Microbiologists, Vancouver, B.C., November, 1991
15. ***Jefferies, W.A.,*** Canada Society for Immunology keynote speaker, Montreal, Quebec - The Adenovirus-2 E3/19K protein down regulates the host immune response. 1992.
16. ***Jefferies, W.A.***, Regulation of the Phosphorylation of MHC Class I molecules by the E3 region of Adenovirus Type 2, Canadian Society for Immunology, Lake Louise, Alberta, March 1993.
17. ***Jefferies, W.A.***, Molecular analysis of the F4/80 antigen, Canadian Society for Immunology, Lake Louise, Alberta, March 1993.
18. ***Jefferies, W.A.,*** Expression of recombinant forms of Immunophillins in Baculovirus, Canadian Society for Immunology, Lake Louise, Alberta, March 1993.
19. ***Jefferies, W.A.,*** TAP-1 is involved in peptide transport in the TAP-2-deficient RMA-S cell line. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.
20. ***Jefferies, W.A.,*** The Adenovirus protein E3/19K exhibits chaperone-like behavior in the endoplasmic reticulum. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.
21. ***Jefferies, W.A.,*** A novel TAP transporter is peptide selective. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.
22. ***Jefferies, W.A.,*** Screening hybridomas by fluorescence concentration analysis. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.
23. ***Jefferies, W.A.,*** Restricted isoform expression and tyrosine phosphatase activity of CD45 in murine dendritic cells. Canadian Society for Immunology, Lake Louise, Alberta, March 1995.
24. ***Jefferies, W.A.,*** Characterization of novel adenoviral protein and its ability to modulate MHC class I surface expression. Canadian Society for Immunology, Lake Louise, AB, March 1995.
25. ***Jefferies, W.A.,*** Kennard, M.L., Gabathuler, R., Food, M. R., Yamada, T., McGeer, P. Studies on the melanotransferrin molecule. National Centre of Excellence, Neural regeneration Network, June, 1995, St. Adéle, Que.
26. ***Jefferies, W.A.,*** Determination of the TAP transporters' specificity in the viral processing and presentation deficient cell line CMT.64. 23rd Meeting of the Federation of European Biochemical Societies, Basel, Suisse, August, 1995.
27. ***Jefferies, W.A.,*** Characterisation of a GPI-linked protein, Melanotransferrin (p97), involved in transferrin-indepedent uptake of iron. 23rd Meeting of the Federation of European Biochemical Societies, Basel, Suisse, August, 1995.
28. ***Jefferies, W.A.,*** Tap-1 is sufficient for the transport of selected peptides. 23rd Meeting of the Federation of European Biochemical Societies, Basel, Suisse, August, 1995.
29. ***Jefferies, W.A.,*** Convenor ASM Meeting, Newport, Oregon, Persistence mechanisms of Viruses.
30. ***Jefferies, W.A.,*** Serum levels of the Iron Binding Protein p97: A novel biological marker of Alzheimer's Disease. Fifth International conference Disease and related disorders, Osaka Japan, 24th July, 1996
31. ***Jefferies, W.A.,*** Molecular Biology of Alzheimer's Disease, IBC Conference San Francisco, California, p97 and Alzheimer's Disease 1997
32. ***Jefferies, W.A.***, Serum p97 levels as a screening test for Alzheimer's disease. 6th International conference on Alzheimer's Disease and Related Disorders Amstersdam, The Netherlands 18-23 July, 1998
33. ***Jefferies, W.A,*** Human melanotransferrin binds to the human transferrin receptor in vitro. BioIron '99, World Congress on Iron Metabolism. Naples, Italy, May 1999
34. ***Jefferies, W.A,*** Metal binding properties of the novel iron binding protein, melanotransferrin (p97). BioIron '99, World Congress on Iron Metabolism. Naples, Italy, May 1999

35. ***Jefferies, W.A.,*** Transcytosis of p97 across the blood-brain barrier. American Society for Pharmacologists. New Orleans, Louisiana, November 1999
36. ***Jefferies, W.A.,*** Round Table discussion on Biomarkers of Alzheimer's Disease International Congress of Alzheimer's and related diseases, Amsterdam 1998
37. ***Jefferies, W.A.,*** Toward a Comprehensive Theory for Alzheimer's Disease Orlando Florida, AD Association 1998
38. ***Jefferies, W.A.,*** Gordon Conference, Barriers of the Central Nervous System. New Hampshire, USA. 2002
39. ***Jefferies, W.A.,*** Annual Canadian Vaccine Meeting. Toronto, Ont., Canada. 2002
40. ***Jefferies, W.A.,*** Progress in Vaccination against Cancer (PIVAC). British Society for Immunology. Nottingham, England. 2002
41. ***Jefferies, W.A.,*** Keystone Conference – Tumor Immunology Keystone, Colorado 2003
42. ***Jefferies, W.A.,*** Keystone Conference – Tumor Immunology II. Keystone, CO 2005
43. ***Jefferies, W.A.,*** 9th International Symposium on Dendritic Cell - Edinburgh, Scotland (delivered by graduate student Robyn Seipp) September 2006
44. ***Jefferies, W.A.,*** Vaccines on the Horizon - 7th Canadian Immunization Conference, Winnipeg, MB 2006
45. ***Jefferies, W.A.,*** Prostate Cancer Research Retreat, Canadian Prostate Cancer Research Foundation, Oakley, ON (delivered by graduate student Francesca Setiadi). February 2007
46. ***Jefferies, W.A.,*** Vaccine Evaluation Centre, Vancouver, BC April 2007
47. ***Jefferies, W.A.,*** Keystone Conference – Cancer Vaccines. Banff, AB March/April 2007
48. ***Jefferies, W.A.,*** Cerebral Vascular Biology Conference, Ottawa, ON June 2007
49. ***Jefferies, W.A.,*** Gordon Conference, Antigen Cross-Presentation, Big Sky Montana, Sept 2-7, 2007
50. ***Jefferies, W.A.*** Vaccine Congress, Amsterdam, The Netherlands, December 9-11, 2007 (delivered by graduate student Robyn Seipp)
51. ***Jefferies, W.A.*** Canadian Society for Immunology and Cancer Immune Therapy Symposium, Whistler, BC Canada, April 3-6, 2009, Tumor Immune Therapy: Trials and Tribulations

10. SERVICE TO THE UNIVERSITY

(a) Memberships on committees, including offices held and dates

1. 1990-2001: Founder and Organizer of the Campus Wide Immunology Study Group
2. 1990-present: Member the University of British Columbia Biochemical Discussion Group
3. 1990-present: Member the University of British Columbia Immunology Seminar Group
4. 1991: Chairman, Department of Microbiology, Pathogenesis Course Evaluation Committee
5. 1991: Member, Department of Microbiology, Immunology Course Evaluation Committee
6. 1991: Member, Department of Microbiology, Cell Biology Course Evaluation Committee
7. 1991: Member, Department of Microbiology, Immunology Curriculum Committee
8. 1992: Medicine 2000 Immunology/Transplantation Organizing Committee
9. 1992: Secretary Biotechnology Safety Committee
10. 1993: Member, Adhoc Committee, Microbiology 200

11. 1993: Secretary Biotechnology Safety Committee
12. 1993: Member, Undergraduate Genetic courses Evaluation Committee, Medical Genetics
13. 1995-2001: Graduate Council Representative for the Department of Microbiology and Immunology
14. 1996: Chairman Biotechnology Laboratory Awards Committee
15. 1997: Faculty of Science committee for Dissertation Gold Medal
16. 1998: Search Committee, Biotechnology Laboratory, Instructor position
17. 1998: Tenure Committee, Microbiology and Immunology, Michael Gold
18. 1998: Search Committee, Microbiology and Immunology, Virology Position
19. 1998: Committee to review B.C. Biotechnology Alliance High School Research Applications
20. 1998-2000: Director, the University of British Columbia. Centre for Molecular Immunology
21. 2000-present: Principal Investigator, CANVAC
22. 1998-2000: Organizing Campus Wide Transgenic Animal Facility
23. 2000-present: Director of the University of British Columbia Transgenic and Knockout Facility
24. 2000-present: Member of the University of British Columbia Animal Users Committee
25. 2004-present: Director of the University of British Columbia Rederivation Facility
26. 2005-present: Member of TRID (CHIR-UBC Strategic Training Program for Translational Research in Infectious Diseases) Faculty Member
27. 2006-present: Director of the TransArc Network
28. 2006-present: Internal Grant Reviewer for Faculty of Science and Faculty of Medicine
29. 2006-present: Member of the Centre for Drug Research and Development
30. 2006-present: Centre for Blood Research at the University of British Columbia, Faculty Member
31. 2007-present: Co-Executive Director of the BC Pre-Clinical Research Consortium (BC-PRC)
32. 2008: Tenure Committee, Zoology, University of British Columbia, Nelly Pante
33. 2009: Tenure Committee, Microbiology & Immunology, University of British Columbia, Marc Horwitz

(b) Other service, including dates

1. 1989: The University of British Columbia Program for Effective Teaching Methods -One day seminar
2. 1991: The University of British Columbia Program for Effective Teaching Techniques-Three day seminar
3. 2002: The University of British Columbia Transgenic Animal Techniques Course offered to research com

11. SERVICE TO THE COMMUNITY

(a) Memberships on scholarly societies, including offices held and dates

1. 1982- present British Biochemical Society
2. 1982-1989 British Transplantation Society
3. 1982- present British Society of Immunology
4. 1990-present Canadian Society of Immunology
5. 1998-2000 Director of the Centre for Molecular Immunology
6. 2001-2003 BC Biotechnology Awards Committee, Chairman
7. 2005-2007 NRC Advisory Board, Ottawa, Canada
8. 2008-present -Faculty of 1000

(b) Memberships on scholarly committees, including offices held and dates

1. Member of the Ronald and Nancy Alzheimer's Research Association Committee on Emerging Biomarkers for Alzheimer's Disease
2. "The Consensus Report of the Working Group on: 'Molecular and Biochemical Markers of Alzheimer's Disease,' which appears in the April 1998 issue of the journal Neurobiology of Aging.
3. 1991-92 National Cancer Institute of Canada fellowship, scholarship, and career award panel member
4. 1992-93 National Cancer Institute of Canada, Immunology Grants Committee
5. 1993-94 National Cancer Institute of Canada, Immunology Grants Committee
6. 1998-2001 Canadian Institutes for Health Research Immunology Panel
7. 2000-2004 Alberta Heritage Fund and Career Award committee
8. 2005-2009 HERRO Program of Mentoring Faculty of the University of British Columbia

(d) Memberships on other committees, including offices held and dates

e) Editorships (list journal and dates)

1. 2000-2001 International Journal of Cancer, Editor
2. Jan 2001-Dec 2002: Journal of Alzheimer's Disease, Associate Editor

(f) Reviewer (journal, agency, etc. including dates)

Peer Reviews: Grants

1. 1990 - Natural Science and Engineering Research Council of Canada Operating grant
2. 1990 - Natural Science and Engineering Research Council of Canada Strategic grant
3. 1991 - Natural Science and Engineering Research Council of Canada Strategic grant

4. 1991 - Medical Research Council of Canada Operating grants
5. 1991/92 - Medical Research Council of Canada grants
6. 1991 - St. Paul's Hospital Foundation Grant
7. 1992- National Cancer Institute of Canada, Career Fellowships
8. 1992- Alberta Cancer Society grant
9. 1992-93 - Medical Research Council of Canada Operating grants
10. 1992-93 - National Cancer Institute of Canada Operating grants
11. 1993-94 - Medical Research Council of Canada Operating grants
12. 1993-94 - National Cancer Institute of Canada Operating grants
13. 1994- Alberta Cancer Society grant
14. 1996 Medical Research Council of Canada Operating grants
15. 1997 Medical Research Council of Canada Operating grants
16. 1997- Manitoba Research Foundation
17. 1998 Medical Research Council of Canada Operating grants
18. 1998- Manitoba Research Foundation
19. 1998- Dalhousie Research Foundation
20. 1998- Heart and Stoke Foundation
21. 2000-02 Canadian Network for Vaccines and Immunotherapeutics of Cancer and Chronic Viral Disease
22. 2001-02 BCRP Concept Award: U.S. Army Medical Research Acquisition Activity
23. 2001-04 National Cancer Institute
24. 2001-02 CANFAR
25. 2005-09 CIHR

Peer Reviews: Journals

1. FEBS Letters
2. International Immunology
3. Biochem Biophys Acta
4. Journal of Neuroscience
5. Blood
6. Journal of Leukocyte Biology
7. Pharmacology, Biochemistry and Behavior

8. European Journal of Immunology
9. Nature Medicine
10. FASEB
11. Journal of Immunology
12. Nature Immunology
13. Cancer Research
14. Journal of Alzheimer Disease
15. International Journal of Cancer

National Grant Review Panels

1. 1992 National Cancer Institute of Canada Career Award and Fellowship Panel
2. 1993 National Cancer Institute of Canada Immunology Panel
3. 1994 National Cancer Institute of Canada Immunology Panel
4. 1999-2000 Canadian Institutes of Health Research, Immunology and Transplantation Panel
5. 2006 Canadian Institutes of Health Research, Immunology and Transplantation Panel
4. 2007 Canadian Institutes of Health Research, Immunology and Transplantation Panel

(g) External examiner:

1. Mark Luscher Ph. D., University of Toronto, Brian Barber (supervisor) 2004
2. Jay C. Varghese Ph.D. University of Alberta, Kevin Kane (supervisor) 2008

(h) Consultant (indicate organization and dates)

1. 1997-2003: Scientific Consultant, Synapse Technologies Incorporated.
2. 2000-2007: Scientific Consultant, TapImmune Incorporated
3. 2008-present: Scientific Consultant , biOasis Technologies Incorporated

(i) Other service to the community

1. 1990: Host of the EUCLID recipients, Award winners of the province wide Math prizes
2. 1990: Host Organizer/Lecturer of the University of British Columbia Connect 90' B.C. High School Science Student Forum
3. 1990: Faculty Representative at the Convocation Ceremonies for the Faculty of Science

4. 1990: Faculty of Science, Open House Display

5. 1991: Lecturer: Three day seminar the University of British Columbia Connect 91' B.C. High School Science Student Forum

6. 1991: Judge for the Canada Wide Science Fair for Canadian High School Students

7. 1991: Faculty Representative at the University of British Columbia. Convocation Ceremonies for the Faculty of Science

8. 1991: OUTREACH Volunteer the University of British Columbia participation in High School Graduation Ceremonies

9. 1992: Faculty Representative at the University of British Columbia Convocation Ceremonies for the Faculty of Science

10. 1992: University sponsored lecture: Shad Valley Program

11. 1993: Faculty Representative at the University of British Columbia. Convocation Ceremonies for the Faculty of Science

12. 1993: University sponsored lecture: Shad Valley Program for exceptional B.C. High school students

13. 1993-Present: Lecturer: Three day seminar the University of British Columbia ***Connect*** B.C. High School Science Student Forum

14. 1998: Supervised High School Co-op Research Projects

15. 1994 - present: Soccer Coach for over 30 teams in the Provincial league.

12. AWARDS AND DISTINCTIONS

(a) Awards for Scholarship

1. 1985 Lady Tata Memorial Leukemia Fellowship

2. 1985 Royal Society of London Postdoctoral Fellowship

3. 1985-86 Welcome Trust Postdoctoral Fellowship

4. 1986-89 Medical Research Council of Canada Postdoctoral Fellowship

5. 1990 University Nominated for Hughes Institutes for Medical Research Centre

6. 1994 Named Principal Investigator for the National Network of Excellence for Neural Regeneration

7. 1995 University Nominated for Hughes Institute for Medical Research International Scholarship
8. 2001-2002 University Killam Faculty Research Fellowship
9. 2008: Wiederhelm Award, The Federation of American Societies for Experimental Biology

(c) Other Awards

Academic awards and distinctions (prior to final degree)

1. 1980 British Columbia Cancer Research Summer Scholarship
2. 1982-1985 Overseas Research Student Scholarship (British)
3. 1982-84 British Columbia Health Care Trainingship Award
4. 1982 Natural Science and Engineering Council of Canada Graduate Scholarship
5. 1982-85 Royal Commission for Exhibition of 1851 Scholarship (London)

Publications Record

Date: Sept 13, 2009

SURNAME: JEFFERIES **FIRST NAME:** Wilfred **MIDDLE NAME(S):** Arthur

	Summary of Refereed Publications		Summary of Non-Refereed Publications		
	1A *(journals)*	1B *(reviews)*	2 *(conf. pro.)*	3 *(books)*	4 *(patents)*
Career	71	8	100	5	40
Last 5 Years	20	2	25	2	4

1. REFEREED PUBLICATIONS

(a) Journals (* denotes publications of primary importance)

1. ***Jefferies WA**, Brandon MR, Hunt SV, Williams AF, Gatter KC, Mason DY. (1984) Transferrin receptor on endothelium of brain capillaries. **Nature** 312: 162-163.

2. **Jefferies WA**, Brandon MR, Williams AF, Hunt SV. (1985) Analysis of lymphopoietic stem cells with a monoclonal antibody to the rat transferrin receptor. **Immunology** 54: 333-341.

3. **Jefferies WA**, Green JR, Williams AF. (1985) Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. **J. Exp. Med.** 162: 117-127.

4. Arvieux J, **Jefferies WA**, Paterson DJ, Williams AF, Green JR. (1986) Monoclonal antibodies against a rat leukocyte antigen block antigen-induced T-cell responses via an effect on accessory cells. **Immunology** 58: 337-342.

5. ***Jefferies WA**, Barclay AN, Gagnon J, Williams AF. (1986) Structure of the CD4(W3/25) T-helper lymphocyte glycoprotein. **Biochemical Society Transactions** 14(2): 336.

6. Paterson DJ, Green JR, **Jefferies WA**, Puklavec M, Williams AF. (1986) The MRC OX-44 antigen marks a functionally relevant subset among rat thymocytes. **J. Exp. Med.** 165: 1-13.

7. *Clark SJ, **Jefferies WA**, Barclay AN, Gagnon J, Williams AF. (1987) Peptide and nucleotide sequences of rat CD4(W3/25) antigen: evidence for derivation from a structure with four immunoglobulin-related domains. **Proc. Natl. Acad. Sci. U.S.A.** 84: 1649-1653.

8. Crocker PR, **Jefferies WA**, Clark SJ, Chung PL, Gordon S. (1987) Species heterogeneity in macrophage expression of the CD4 antigen (AIDS virus receptor). **J. Exp. Med.** 166: 613-618.

9. **Jefferies WA** and MacPherson GG. (1987) Expression of the W6/32 HLA class I epitope by cells of rat, mouse, human and other species: critical dependence on the interaction of specific MHC heavy chains with human and bovine beta 2-microglobulin. **Eur. J. of Immunol,** 17: 1257-1263.

10. Paterson DJ, **Jefferies WA**, Green JR, Brandon MR, Corthesy P, Puklavec MJ, Williams AF. (1987) Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T cell blasts. **Molecular Immunology** 24: 1281-1290.

11. ***Jefferies WA**, Ruther U, Wagner EF, Kvist S. (1988) Cytolytic T cells recognize a chimeric MHC class I antigen expressed in influenza A infected transfenic mice. **EMBO Journal** 7: 3423-3431.

12. **Jefferies WA** and Burgert H-G. (1990) E3/19k from Adenovirus 2 is an immunosubversive protein that binds to a structural motif regulating the intracellular transport of major histocompatibility complex class I proteins. **J.Exp. Med.** 172: 1653-1664.

13. Lippé R, Luke E, Kauh Y-T, Lomas C, **Jefferies WA.** (1991) Adenovirus infection inhibits the phosphorylation of major histocompatibility complex class I proteins. **J. Exp. Med.** 174: 1159-1166.

14. Matsuse T, Hayashi S, Kuwano K, Keunecke H, **Jefferies WA**, Hogg JC. (1992) Latent adenoviral infection in the pathogenesis of chronic airways obstruction. **Am. Rev. Respir Dis.** 146: 177-184.

15. Michaelis C, Banfield B, Gruenheid S, Tsang Y, Lippé R, **Jefferies WA**, Wattenberg B, Tufaro F. (1992) Toxin resistance and reduced secretion in a mouse L-cell mutant defective in Herpes Virus propagation. Biochemistry and Cell Biology, anniversary edition. **Biochem and Cell Biol.** 70: 1209-1217

16. Ou D, Chong P, Choi Y, McVeigh P, **Jefferies WA**, Gerasimos K, Tingle A.J, Gillam S. (1992) Identification of T-cell epitopes on E2 protein of rubella virus, as recognized by human T-cell lines and clones. **J. Virology** 66: 6788-6793.

17. Ou D, Chong P, McVeigh P, **Jefferies WA**, Gillam S. (1992) Characterization of the specificity and genetic restriction of human CD4+ cytotoxic T cell clones reactive to capsid antigen of rubella virus. **Virology** 191: 680-686.

18. Kennard M, Food M, **Jefferies WA**, Piret J. (1993) Controlled release process to recover heterologous glycosylphosphatidylinositol anchored proteins from CHO cells. **Biotechnology and Bioengineering** 42: 480-486.

19. Lippé R, Kolaitis G, Michaelis C, Tufaro F, and **Jefferies WA.** (1993) Differential recruitment of viral and allo-epitopes into the MHC Class I antigen processing pathway of a novel mutant of Ltk^- cells. **J. Immunol.** 150: 3170-3179.

20. **Jefferies WA**, Kolaitis G, Gabathuler R. (1993) The Interferon-gamma-induced recognition of the antigen-processing variant CMT.64 by cytolytic T cells can be replaced by sequential addition of beta2 microglobulin and antigenic peptides. **J. Immunol.** 151: 2974-2985.

21. *Food M, Rothenberger S, Gabathuler R, Haidl I, Reid G, **Jefferies WA.** (1994) Transport and expression in human melanomas of a transferrin-like glycosylphosphatidylinositol anchored protein. **J. Biol. Chem.** 269: 3034-3040.

22. Stokes R, Haidl I, **Jefferies WA,** Speert D. (1993) Mycobacteria-macrophage interactions. **J. Immunol.** 151: 7067-7076.

23. Brennan J, Mager D, **Jefferies WA,** Takei F. (1994) Expression of different members of the Ly-49 gene family defines distinct natural killer cell subsets and cell adhesion properties. **J. Exp. Med.** 180: 2287-2295.

24. *Gabathuler R, Reid G, Kolaitis G, Driscoll J, **Jefferies WA.** (1994) Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. **J. Exp. Med.** 180: 1415-1425.

25. Garcia-del Portillo F, Pucciarelli MG, **Jefferies WA,** Finlay BB. (1994) Salmonella typhimurium induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells. **J. Cell Science** 107: 2005-2020.

26. *Kennard M, Richardson D, Gabathuler R, Ponka P, **Jefferies WA.** (1995) A novel iron uptake mechanism mediated by GPI-anchored human p97. **EMBO Journal** 14: 4178-4186.

27. Haidl I, Rothenberger S, Johnson P, **Jefferies WA.** (1995) Detection of restricted isoform expression and tyrosine phosphatase activity of CD45 in murine dendritic cells. **Eur. J. Immunol.** 25: 3370-3374.

28. * Rothenberger S, Food MR, Gabathuler R, Kennard M, Yamada T, Yasuhara O, McGeer PL, **Jefferies WA.** (1996) Coincident expression and distribution of melanotransferrin and transferrin receptor in human brain capillary endothelium. **Brain Research** 712:117-121.

29. **Jefferies WA,** Food MR, Gabathuler R, Kennard M, Rothenberger S, Yamada T, Yasuhara O, McGeer PL. (1996) Reactive microglia specifically associated with amyloid plaques in Alzheimer's disease brain tissue express melanotransferrin. **Brain Research** 712:122-126.

30. *Kennard M, Feldman H, Yamada T, **Jefferies WA.** (1996) Serum levels of the iron binding protein p97 are elevated in Alzheimer's disease. **Nature Medicine** 11:1230-1235.

31. Haidl I, and **Jefferies WA.** (1996) The macrophage cell surface glycoprotein F4/80 is a highly glycosylated proteoglycan. **Eur. J. Immunol.** 26:1139-1146.

32. Brennan J, Mahon G, Mager DL, **Jefferies WA,** Takei F. (1996) Recognition of Class I major histocompatibility complex molecules by Ly-49: specificities and domain interaction. **J. Exp. Med.** 183:1553-1559.

33. Marusina K, Reid G, Gabathuler R, **Jefferies WA,** Monaco JJ. (1997) Novel peptide-binding proteins and peptide transport in normal and TAP-deficient microsomes. **Biochemistry** 36: 856-863.

34. Shimizu K, Kennard M, Gabathuler R, Rothenberger S, Theilmann D, **Jefferies WA.** (1997) Expression of cell surface GPI-anchored human p97 in baculovirus-infected insect cells. **Biotech. Bioeng.** 55: 1-12.

35. Yamada T, Yoshiyama Y, Kawaguchi N, Ichinose A, Iwaki T, Hirose S, **Jefferies WA.** (1998) Possible roles of transglutaminases in Alzheimer's disease. **Dement Geriatr Cogn Disord,** 9: 103-110.

36. Gabathuler R, Alimonti J, Zhang QJ, Kolaitis G, Reid G, **Jefferies WA.** (1998) Surrogate antigen processing mediated by TAP-dependent antigenic peptide secretion. **Journal of Cell Biology** 140: 17-27.

37. Hmama Z, Gabathuler R, **Jefferies WA**, De Jong G, Reiner NE. (1998) Attenuation of HLA-DR expression by mononuclear phagocytes infected with Mycobacterium tuberculosis is related to intracellular sequestration of immature Class II heterodimers. **J. Immunol.** 161: 4882-4893.

38. Hegedus D, Pfeifer T, Grigliatti T, Theilman D, Gabathuler R, Kennard ML, **Jefferies WA.** (1999) Differences in the expression and the localization of human melanotransferrin in lepidopteran and depteran insect cell lines. **Protein Expression & Purification** 15: 296-307.

39. Yamada T, Tsujioka Y, Taguchi T, Takahashi M, Tsuboi Y, Moroo I, Yang J, **Jefferies WA.** (1999) Melanotransferrin is produced by senile plaque-associated reactive microglia in Alzheimer's disease. **Brain Research** 845: 1-5.

40. Moroo I, Yamada T, Gabathuler R, Kennard ML, Nurminen J, **Jefferies WA.** (1999) Use of p97 in the diagnosis of Alzheimer's disease. **Alzheimer's Report** 2: 353-358.

41. *Alimonti J, Zhang QJ, Gabathuler R, Reid G, Chen S, **Jefferies W.A.** (2000) TAP expression provides a general method for improving the recognition of malignant cells in vivo. **Nature Biotechnol** 18:515-520.

42. Zhang QJ. Chen S, Saari C, Massuci M, Tufaro F, and **Jefferies W.A.** (2000) Evidence of selective processing of immunodominant epitopes in virally infected cells. **J. Immunol** 164:4513-4521.

43. * Feldman, H., Gabathuler, R., Kennard, M., Nurminen, J., Levy, D., Foti, S., Foti, D., Beattie, B.l., **Jefferies, W.A.** (2001). Serum p97 levels as an aid to identifying Alzheimer's disease. **J Alzheimer's Disease.** 3: 507-516.

44. Moise A.R., Grant J.R., Vitalis T. Z. and **Jefferies,W. A.** (2002) Adenovirus E3-6.7K Maintains Calcium Homeostasis and Prevents Apoptosis and Arachidonic Acid Release, **J.Virology.** 76(4): 1578-1587.

45. * Sala R., **Jefferies W.A.,** Walker B., Yang J., Tiong J., Law S. K. A., Carlevaro F., Di Marco E., Vacca A., Cancedda R., Descalzi Cancedda F., Rabatti D. (2002) The human melano-associated protein melanotransferrin promotes endothelial cell migration and angiogenesis in vivo. **Eur J Cell Biol** 81:1-9.

46. Demeule M, Poirier J, Jodoin J, Bertrand Y, Desrosiers RR, Dagenais C, Nguyen T, Lanthier J, Gabathuler R, Kennard M, **Jefferies WA**, Karkan D, Tsai S, Fenart L, Cecchelli R, Beliveau R. (2002) Links High transcytosis of melanotransferrin (P97) across the blood-brain barrier. **J Neurochem**. Nov;83(4):924-33.

47. * Moroo I., Ujiie M., Walker B. L., Tiong J. W. C., Vitalis T. Z., Karkan D., Gabathuler R., Moise A. R., and **Jefferies W.A.** (2003) Identification of a novel route of iron transcytosis across the mammalian blood-brain barrier. **Microcirculation** 10, 457-462.

48. Kotturi M.F., Carlow DA, Lee JC, Ziltener HJ, and **Jefferies W.A.** (2003). Identificatioon and Functional Characterization of Voltage-dependent Calcium Channels in T lymphocytes. **J Biol Chem** Nov 21; 278(47): 46949-60.

49. * Ujiie M., Dickstein D., Carlow D., and **Jefferies, W.A.** (2003) Blood-brain barrier permeability precedes senile plaque formation in an Alzheimer disease model. **Microcirculation** 10, 463-470.

50. * Lizée G., Basha G., Tiong J., Julien JP., Tian M., Biron KE., and **Jefferies, W.A.** (2003) Control of dendritic cell cross-presention by the major histocompatibility complex class I cytoplasmic domain. **Nature Immunol.** Nov.4:11; 1065-1073.

51. Yang J., Tiong J., Kennard, M., and **Jefferies W.A.** (2004) Deletion of the GPI Pre-Anchor Sequence in Human p97- a General Approach for Generating the Soluble Form of GPI-Linked Proteins. **Protein Expr. Purif.** Mar;34(1):28-48.

52. Moise AR, Grant J, Lippe R, Gabuthuer R, and Jefferies WA. (2004) The adenovirus E3-6.7K protein adopts diverse membrane topologies following posttranslational translocation. **J Virol** 78 (1): 454-63.

53. Leitch J, Fraser K, Lane C, Putzu K, Adema GJ, Zhang QJ, **Jefferies WA**, Bramson JL, Wan Y. (2004) CTL-dependent and –independent antitumor immunity is determined by the tumor not the vaccine. **J. Immunol.** 172 (9): 5200-5

54. Setiadi AF, David MD, Chen SS, Hiscott J, **Jefferies WA**. (2005) Identification of Mechanisms Underlying TAP Deficiency in Metastatic Murine Carcinomas. **Cancer Res. (Aug 15) 65 (16):7485-92.**

55. Yuanmei Lou, Timothy Z. Vitalis, Bing Cai, Barbara Seliger, Derek Atkins, Susan S. Chen, Andrew P. Jeffries, Genc Basha, Kyung Bok Choi, **Wilfred A. Jefferies**. (2005) The restoration of TAP1 expression in lung carcinoma increases dendritic cell cross-priming, tumor specific immune responses and survival. **Cancer Research (Sept 1) 65 (17):7926-33.**

56. Kotturi Maya F., and **Jefferies W.A.** (2005) Splice conversion results in Novel Chimeric Splice Isoforms of an L-Type Voltage-Dependent Calcium Channel in T Lymphocytes. **Mol Immunol. 42(1):1461-74.**

57. Creagh Louise A., Tiong Jacqueline W.C., Tian Mei Mei, Haynes Charles A. and **Jefferies Wilfred A.** (2005) Calorimetric studies of melanotransferrin (p97) and its interaction with iron. **J. Biol. Chem Apr 22;280(16):15735-41 (Epub 2005 Feb9).**

58. Kotturi MF, **Jefferies WA.** (2005) Molecular characterization of L-type calcium channel splice variants expressed in human T-lymphocytes. **Mol. Immunol. Aug. 24(12):1461-74.**

59. * Vitalis TZ, Zhang QJ, Alimonti J, Chen SS, Basha G, Moise A, Tiong J, Tian MM, Choi KB, Waterfield D, Jeffries A, **Jefferies WA.** (2005) Using the TAP Component of the Antigen-Processing Machinery as a Molecular Adjuvant. **PLoS Pathog. Dec;1(4):e36. Epub 2005 Dec 30.**

60. Lou Y, Vitalis TZ, Basha G, Cai B, Chen SS, Choi KB, Jeffries AP, Elliott WM, Atkins D, Seliger B, **Jefferies WA.** (2005) Restoration of the expression of transporters associated with antigen processing in lung carcinoma increases tumor-specific immune responses and survival. **Cancer Res. Sep 1;65(17):7926-33.**

61. * Dickstein DL, Biron KE, Ujiie M, Pfeifer CG, Jeffries AR, **Jefferies WA.** (2006) Amyloid beta peptide immunization restores blood-brain barrier integrity in Alzheimer disease. **FASEB J. Mar;20(3):426-33.**

62. * Lou Yuanmei, Seipp RP,Cai B, Chen SS, Vitalis TZ, Choi KB, Pearson TW, Jeffries AP, Gopaul RS, Li X, Seliger B, and **Jefferies W.A. (2007)** Increased Antigen Presentation, Decreased Melanoma Tumor Growth and Production of Anti-Tumor T Cell Memory by Recombinant Adenovirus Encoding TAP1. **Vaccine. Mar 8;25(12):2331-9. Epub 2006 Dec 12.**

63. Zhang QJ, Seipp RP, Chen SS, Vitalis TZ, Li XL, Choi KB, Jeffries A, **Jefferies WA. (2007)** TAP expression reduces IL-10 expressing tumor infiltrating lymphocytes and restores immunosurveillance against melanoma. **Int J Cancer. May 1;120(9):1935-41.**

64. * Grant JR, Moise AR, and **Jefferies WA. (2007)** Identification of a novel immunosubversion mechanism by a virologue of B lymphocyte TACI, **Clin Vaccine Immunol. Jul;14(7):907-17**

65. A. Francesca Setiadi, David MD, Seipp RP, Hartikainen JA, Gopaul R, **Jefferies WA. (2007)** Epigenetic control of the immune escape mechanisms in malignant carcinomas. **Mol. Cell. Biol., Nov. 27(22): 7886-7894.**

66. Lou Y, Basha G, Seipp RP, Cai B, Chen SS, Moise AR, Jeffries AP, Gopaul RS, Vitalis TZ, **Jefferies WA. (2008)** Combining the antigen processing components TAP and Tapasin elicits enhanced tumor-free survival.**Clin Cancer Res. 2008 Mar 1;14(5):1494-501.**

67. Karkan D, Pfeifer C, Vitalis TZ, Arthur G, Ujiie M, Chen Q, Tsai S, Koliatis G, Gabathuler R, **Jefferies WA. (2008)** A unique carrier for delivery of therapeutic compounds beyond the blood-brain barrier. **PLoS ONE. Jun 25;3(6):e2469.**

68. Zhang QJ, Li XL, Wang D, Huang XC, Mathis JM, Duan WM, Knight D, Shi R, Glass J, Zhang DQ, Eisenbach L, and **Jefferies WA. (2008)** Trogocytosis of MHC-I/peptide Complexes Derived from Tumors and Infected Cells Enhances Dendritic Cell Cross-priming and Promotes T Cell Responses. **PLoS ONE. Aug 29;3(8):e3097.**

69. Basha G, Lizée G, Reinicke AT, Seipp RP, Omilusik, KD and **Jefferies WA. (2008)** MHC Class I Endosomal and Lysosomal Trafficking Coincides with Exogenous Antigen Loading in Dendritic Cells. **PLoS ONE, Sept 19;3(9):e3247.**

70. Setiadi AF, Omilusik K, David MD, Seipp RP, Hartikainen J, Gopaul R, Choi KB and **Jefferies WA. (2008)** Epigenetic Enhancement of Antigen Processing and Presentation Promotes Immune Recognition of Tumors. **Cancer Res., Dec 1;68(23):9601-7.**

71. Reinicke A, Omilusik K, Basha G, ***Jefferies WA.*** Dendritic Cell Cross-priming is Indispensable for Immunity to *Listeria Monocytogenes*. ***PLoS ONE, Oct, 6, 2009 (online)***

(b) Refereed Review

1. **Jefferies WA**, Gabathuler R, Rothenberger S, Food M, Kennard M. (1996) Pumping iron in the 90's. **Trends in Cell Biology 6:223-228.**

2. Feldmann H., Kennard ML, Gabathuler R, Yamada T, Adams S, **Jefferies WA.** (1997) Serum levels of the iron binding protein p97: a novel biological marker of Alzheimer's disease. **In Proceedings of the Fifth International Conference on Alzheimer's disease and related disorders by John Wiley and Sons.**

3. **Jefferies WA,** Lizee G, Kennard ML. (1997) Creation of GPI-anchored fusion proteins. **Methods in Molecular biology, Animal Cell Culture** Humana Press Inc. U.S.A.

4. **Jefferies WA,** (1998) Emerging Biomarkers of Alzheimer's Disease. Working Group on Biological Markers of Alzheimer's Disease, **Alzheimer's Association, Ronald and Nancy Reagan Research Institute and National Institute on Aging, NIH, U.S.A.**

5. Jefferies, W.A., Dickstein, D.L., Ujiie, M. (2001). Assessing p97 as an Alzheimer's disease serum biomarker. **Journal of Alzheimer's Disease. 3:339-344**

6. Ujiie M., Dickstein DL, **Jefferies WA** (2002). P97 as biomarker for Alzheimer disease. **Frontiers in Bioscience. Feb. 1:7:e42-7.**

7. Lizée, G., Basha G., and **Jefferies WA** (2005) Tails of wonder: endocytic-sorting motifs key for exogenous antigen presentation. **Trends in Immunology. Mar;26(3):141-9.**

8. Kotturi, MF, Hunt, SV and **Jefferies, WA.** (2006) Roles of CRAC and Ca(V)-like channels in T cells: more than one gatekeeper? **Trends Pharmaceutical Sciences. Jul;27(7):360-7.**

2. <u>NON-REFEREED PUBLICATIONS</u>

(a) Journals

1. **Jefferies WA.** et al (1998) "Consensus Report of the Working Group on: 'Molecular and Biochemical Markers of Alzheimer's Disease'." **Neurobiology of Aging 4: 1-20.**

2. **Setiadi AF**, David MD, Jefferies WA. Quantitative chromatin immunoprecipitation using the LightCycler 480 system. **Biochemica. 1:8-10, 2008.**

(b) Conference Proceedings

1. **Jefferies WA**, Brandon MR, Hunt SV, Williams AF. Characterization of the rat transferrin receptor. Harden Conference on the Cell Surface Proteins of Lymphocytes, Kent, England, 1982.
2. **Jefferies WA**, Hunt SV, Williams AF. Characterization of the rat receptor for transferrin. Harden Conference on Cell Surface Proteins, Wye College, 1982.
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14 PATENTS

File Date	Country	Status	Patent Title	Patent No.	Issue Date
09/22/1995	Japan	Notice of Allowance	Method of Enhancing Expression of MHC-Class 1 Molecules Bearing Endogenous Peptides		
08/17/2001	PCT	National waived	Chemotherapeutic Agents Conjugated to p97 and Their Methods of Use in Treating Neurological Tumours		
02/08/2001	PCT	National Phase	Compositions and Methods for Screening Therapeutic Agents		
02/23/2001	PCT	National Phase	Novel Circular Extra-Chromosomal DNA Elements		
12/07/2001	PCT	National Phase	CAML Modulation Proteins		
06/07/2000	PCT	National Phase	Apoptosis Inhibition by Adenovirus E3/6.7K		
09/22/1995	PCT	National Phase	Method of Enhancing Expression of MHC-Class I Molecules Bearing Endogenous Peptides		
03/12/1997	PCT	National Phase	Method of Identifying MHC-Class I Restricted Antigens Endogenously Processed By a Cellular Secretory Pathway	WO 97/34143	09/18/1997
08/30/1996	PCT	National Phase	Use of p97 and Iron Binding Proteins as Diagnostic and Therapeutic Agents		
07/09/1993	PCT	National Phase	Use of p97 and Iron Binding Proteins as Diagnostic and Therapeutic Agents		
07/31/2002	Australia	Issued	Compositiona and Methods for Screening Therapeutic Agents	785028	12/14/2006
09/22/1995	European	Issued	Method of Enhancing Expression of MHC-Class 1 Molecules Bearing Endogenous Peptides	0783573	12/21/2005
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03/12/1997	Switzerland	Issued	Method of Identifying MHC-Class I Restricted Antigens Endogenously Processed by a Cellular Secretory Pathway	0888540	07/23/2003
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03/12/1997	European	Issued	Method of Identifiying MHC-Class I Restricted Antigens Endogenously Processed By a Cellular Secretory Pathway	0888540	07/23/2003
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03/12/1997	Italy	Issued	Method of Identifying MHC-Class I Restricted Antigens Endogenously Processed by a Cellular Secretory Pathway	0888540	07/23/2003
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03/12/1997	Sweden	Issued	Method of Identifying MHC-Class I Restricted Antigens Endogenously Processed by a Cellular Secretory Pathway	0888540	07/23/2003
03/12/1997	UK	Issued	Method of Identifying MHC-Class I Restricted Antigens Endogenously Processed by a Cellular Secretory Pathway	0888540	
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08/30/1996	Australia	Issued	Quantitation of p97 to Diagnose and Monitor Alzheimer's Disease	717198	07/06/2000
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02/08/2001	Canada	Filed	Compositions and Methods for Screening Therapeutic Agents		
02/08/2001	European	Filed	Compositions and Methods for Screening Therapeutic Agents		
08/08/2002	Japan	Filed	Compositions and Methods for Screening Therapeutic Agents		
11/30/2006	PCT	Filed	Pox Viridae Treatment		
12/18/2006	Canada	Filed	Anti-Tumour Immunity and Specific T Cell Memory are Induced by Low Dose Inoculation with Nonreplicating Recombinant Adenovirus Encoding TAP1		
01/16/2002	USA	Filed	Method of Enhancing An Immune Response		
07/09/1993	European	Filed	Use of p97 and Iron Binding Proteins as Diagnostic and Therapeutic Agents		
07/09/1993	Canada	Filed	Use of p97 and Iron Binding Proteins as Diagnostic and Therapeutic Agents		
08/30/1996	Canada	Filed	Quantitation of p97 to Diagnose and Monitor Alzheimer's Disease		

Technology Disclosure

1991 Peptides for use in modulation the expression of Human receptors: a general approach.
Co-discoverer: ***W. A. Jefferies***, Mr. G. Kolaitis

Licensed Processed:

1. Beta-emission enhancement solution
Co-discoverer: Drs. ***W. A. Jefferies***, R. Gabathuler
Licensed to Dragon Consultant, May, 1993.

15. WORK SUBMITTED (including publisher and date of submission)

Submitted Journal Articles

1. Seipp, RP, Hoeffel, G, Moise, AR, Lok, S, Ripoche, AC, Marañón, C, Hosmalin, A, and Jefferies, WA. Novel isoforms of tapasin contribute to antigen presentation, ***Submitted to J. Immunology***

2. Johnson L, Zhang F, Basha G, Hartikainen JA, Seipp R, Chen A, Wang R, Ling V, and Jefferies WA. TAPL Orchestrates Dendritic Cell Antigen Sampling and Regulates Lymphocyte Cellularity. ***In revision. Plos ONE***

3. Kotturi M, Cherneski C, Levin A, and Jefferies WA, Immunosuppressive impact of common calcium blockers in renal disease patients. ***Submitted to Hypertension.***

4. Wang YT, Chen X, Omilusik K, Priatel JJ, Choi KB, Gopaul R, Teh HS, Tan R, Bech-Hansen NT, and Jefferies WA. Life, Death and Exhaustion: Tonic Signals Mediated by L-type Ca^{2+} Channels Promote Naïve CD4 and CD8 T Cell Survival. ***Re-submitted to Immunity***.

5. Genc Basha, Kyla Omilusik, Anna Reinicke, Kyung Bok Choi, and Wilfred A. Jefferies An alternative function for the invariant chain in MHC Class I cross presentation and cross-priming in dendritic cells is differentially regulated by distinct cytoplasmic tail motifs. **Submitted to *Science***

6. Dickstein DL, Choi KB, Chung B, Lee J, and Jefferies WA. Amyloid beta regulated expression of melanotransferrin in microglia is inhibited by NSAIDS. ***In revision JBC***

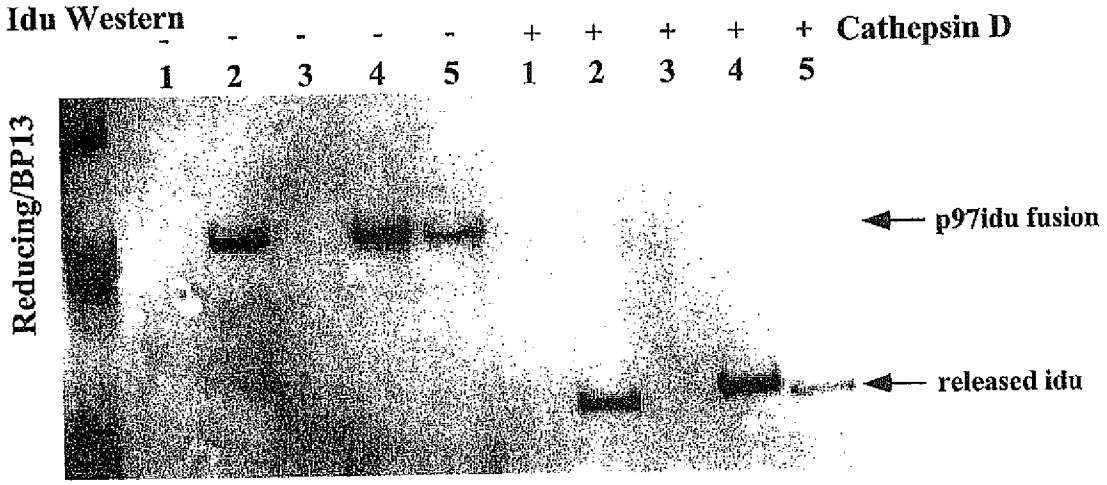
7. Tian MM, Tiong JWC, Martens G, Humphrey EC, Vitalis TZ, and Jefferies WA. Caveolae-dependent endocytosis facitlitate transferrin/transferrin receptor independent iron uptake via GPI-anchored p97 in melanoma cells. ***Submitted to PLoS ONE.***

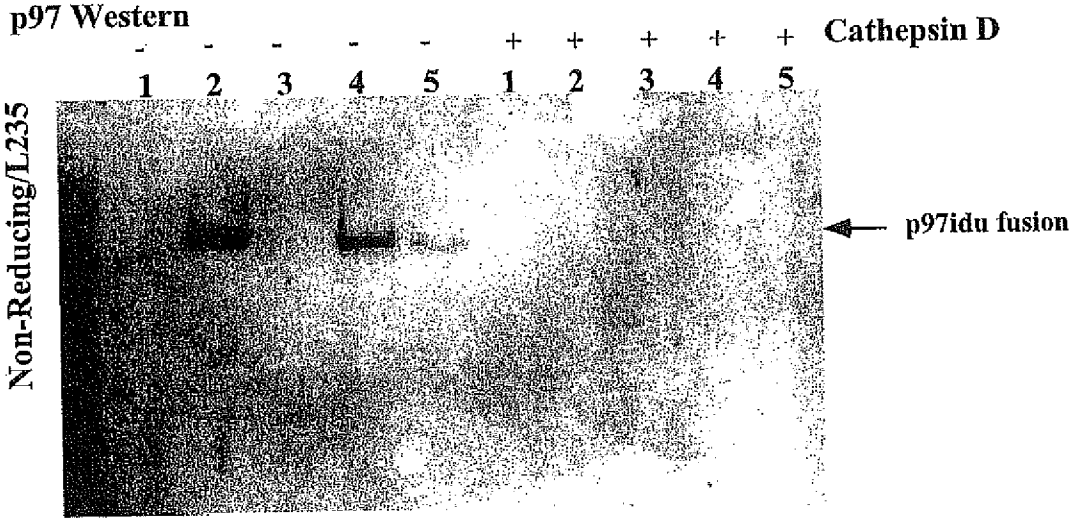
8. Bennett J, Basivireddy J, Kollar A, Biron K, Reickmann P, McNagny K, McQuaid S, Jefferies WA, Abnormal endothelial tight junctions and enhanced vascular permeability of the Blood Brain Barrier in Multiple Sclerosis. ***Draft***

Exhibit B

Effect of Linker on p97idu Fusion Expression and Proteolysis

	linker sequence	4C	37C	37C cathD	Lysate
1	none (empty vector)	0	0	0	0
2	AEAETGSEVNLDAEF	230	280	260	4
3	AEAETGTNFFRD	0	0	0	0
4	AEAETG	470	460	440	8
5	AEAETGKPIEFFRL	110	90	110	2





Conclusions:

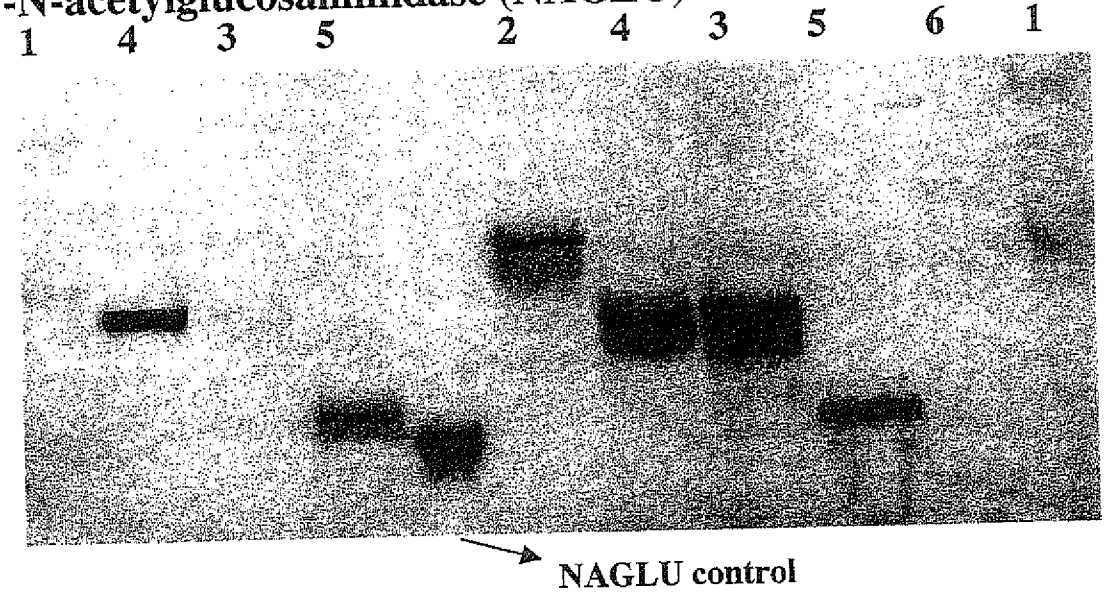
1. **Linker sequence has an effect on fusion expression level.**
2. **Cathepsin D (predominant lysosomal protease) removes p97 from iduronidase *in vitro* without regard for linker. p97 appears to be destroyed in the process.**
3. **Iduronidase activity against the monosaccharide substrate is unaffected by fusion or proteolysis.**
4. **Linker has no effect on lysosomal delivery of the fusion (which is negligible).**

Therefore, the current linker seems to still be the best choice

Exhibit C

Another conjugate of p97

p97-N-acetylglucosaminidase (NAGLU) Fusions



		protein	vector	secreted units/cell
1	Molecular Weight Markers			
2	p97N NAGLU	p97-NAGLU	pC3B	101
3	NAGLU	p97C-NAGLU	pC3B	73
4	p97N NAGLU	p97N-NAGLU	pC3B	234
5	NAGLU	NAGLU	pC3B	133
6		empty vector	pcDNA3.1	0

Exhibit D

Gene Therapy (2007) 14, 523–532

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ORIGINAL ARTICLE

Directing adenovirus across the blood–brain barrier via melanotransferrin (P97) transcytosis pathway in an in vitro model

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Adenovirus serotype 5 (Ad5) is widely used in the development of gene therapy protocols. However, current gene therapy strategies involving brain are mostly based on intracranial injection. A major obstacle for systemically administered vectors to infect brain tissue is the blood–brain barrier (BBB). One strategy to cross the BBB is transcytosis, a transcellular transport process that shuttles a molecule from one side of the cell to the other side. Recently, melanotransferrin (MTf)/P97 was found to be able to cross the BBB and accumulate in brain. We thus hypothesize that re-directing Ad5 vectors to the MTf transcytosis pathway may facilitate Ad5 vectors to cross the BBB. To test this hypothesis, we constructed a bi-specific adaptor protein containing the extracellular domain of the coxsackie-adenovirus receptor (CAR) and the full-length melanotransferrin (sCAR-MTf), and investigated its ability to re-direct Ad5 vectors to the MTf transcytosis pathway. We found this adaptor protein could re-direct Ad5 to the MTf transcytosis pathway in an in vitro BBB model, and the transcytosed Ad5 viral particles retained their native infectivity. The sCAR-MTf-mediated Ad5 transcytosis was temperature- and dose dependent. In addition, we examined the directionality of sCAR-MTf-mediated Ad5 transcytosis, and found the efficiency of apical-to-basal transcytosis was much higher than that of basal-to-apical direction, supporting a role of this strategy in transporting Ad5 vectors towards the brain. Taken together, our study demonstrated that re-directing Ad5 to the MTf transcytosis pathway could facilitate gene delivery across the BBB.
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Keywords: *adenovirus; melanotransferrin; transcytosis; blood–brain barrier; gene therapy*

Introduction

Human adenovirus serotype 5 (Ad5) is widely explored as a gene delivery vector for a variety of tissues/organs including brain, largely owing to its high gene delivery efficiency, easy manipulation, high titer production *in vitro*, well-characterized biology and minor pathological effect in humans. However, for brain-related gene therapy strategies, such as brain cancer gene therapy, and gene therapy for genetic diseases involving central nervous system pathology, current protocols under development are mainly based on intra-cranial injections.[1–3] This is not only clinically difficult because it requires high risk surgical procedures, but also inefficient in gene delivery, as localized injections do not allow Ad5 vectors to access all diseased cells. Therefore, it is essential to develop a strategy that allows systemically administered Ad5 vectors to infect the brain cells.

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A major obstacle for intravascular-administered Ad5 vectors to enter the brain cells is the blood–brain barrier (BBB).[2,3] The BBB is formed by brain capillary endothelial cells, which are closely sealed by tight junctions and contain few fenestrae and few endocytic vesicles. The BBB restricts the transport of most small polar molecules and macromolecules from the cerebrovascular circulation into the brain tissue, including therapeutic agents such as adenoviral vectors.

One strategy to cross the endothelial barrier is transcytosis, a transcellular transport process that shuttles a molecule from one side of the cell to the other side. It involves endocytosis, intracellular transport, and exocytosis of the molecule. Typical examples of this pathway include the transport of albumin and lipids, hormones and peptides that bind avidly to albumin,[4,5] and transferrin receptor (TfR)-mediated transcytosis.[6,7] Transcytosis pathways are not only employed by blood components, but also adopted by viruses such as human immunodeficient viruses, adeno-associated virus and poliovirus.[8–10]

Recent studies discovered that human melanotransferrin (MTf, also named P97), a transferrin homolog originally identified in human melanoma, was highly accumulated into the mouse brain following intravenous injection.[11,12] These studies demonstrated that MTf could

cross the brain endothelial cells without affecting the integrity of the BBB and with a much higher rate than what is seen with transferrin and albumin.[11,12] These studies suggest that MTf could be a good candidate for drug delivery to brain. It should be noted that these studies were performed with human MTf in mouse brain, and in *in vitro* BBB models established with bovine brain microvascular endothelial cells (BBMVEC), suggesting MTf functions without significant species discrepancy.

MTf is a glycosylated protein, and exists in two forms including a cell membrane bound form and a soluble, secreted form.[13] The exact function of both forms remains largely unknown. The membrane bound MTf is composed of 719 amino-acid residues, which comprises two homologous extracellular domains of 342 and 352 amino-acid residues, respectively, and a C-terminal 25-residue stretch of predominantly uncharged and hydrophobic amino-acid residues, which is believed to form a glycosylphosphatidylinositol (GPI) membrane anchor.[13] It has been demonstrated that transendothelial transport of MTf occurs via receptor-mediated endocytosis, and low-density lipoprotein (LDL) receptor-related protein (LRP) appears to be involved in MTf transendothelial transport.[11]

In this study, we attempted to re-direct Ad5 vectors to the MTf transcytosis pathway so that they can deliver transgenes across the BBB in an *in vitro* model system. Infection of Ad5 is initiated by attachment of its capsid protein fiber to the cell surface coxsackie-adenovirus receptor (sCAR), followed by interaction of another capsid protein, penton base, with cell surface α_v integrins that triggers the internalization of the viruses.[14–16] Based on this information, investigators have utilized bi-specific adaptor proteins that bind to both Ad5 vectors and alternative receptors expressed on the surface of the target cells to re-direct Ad5 tropism.[17–19] In our study, we attempted to re-direct Ad5 across the BBB using the bi-specific adaptor protein strategy. We constructed a bi-specific adaptor protein containing the extracellular domain of CAR (sCAR) and the full-length MTf, namely sCAR-MTf, and demonstrated its ability to re-direct Ad5 vectors across the BBB using an *in vitro* BBB model. This work represents the first study that employs transcytosis pathway to re-direct Ad5 vectors across the BBB.

Results

Generation of bi-specific adaptor protein sCAR-MTf

Previous studies have shown that bi-specific adaptor proteins that bind both Ad5 vector and alternative receptors can be used to re-direct Ad5 vector entering cells through the alternative receptors.[17–19] In order to re-direct Ad5 vector to MTf transcytosis pathway, we constructed a bi-specific adaptor protein containing the extracellular domain of CAR and the GPI anchor-deleted full-length MTf (termed sCAR-MTf) (Figure 1a). In the adaptor protein, sCAR that binds to Ad5 fiber is located at the N-terminal end, whereas MTf that targets to MTf transcytosis pathway is located at the C-terminal part. The GPI-deleted MTf was employed because deletion of the GPI anchor has been shown to allow production of the soluble form of MTf.[20] In addition, a bacteriophage T4 fibritin trimerization domain was used to connect the two binding domains, as fiber exists as trimer in Ad5 virions and trimerized sCAR is expected to bind fiber

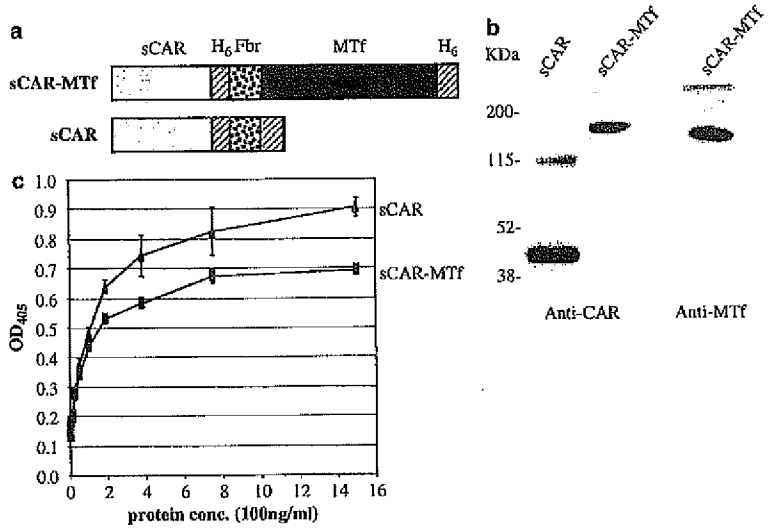

Figure 1 Generation of bi-specific adaptor protein sCAR-MTf. (a) Diagram of the bi-specific adaptor protein sCAR-MTf and control sCAR. H_6: His_6 epitope; Fbr: T4 Fribritin trimerization domain. (b) Western blotting assay showing the adaptor proteins were expressed and purified. In the assay, about 500 ng of each purified protein were used for SDS-PAGE and subsequent Western blotting assay with anti-CAR and/or anti-MTf antibody. Note: the upper fainter band in sCAR lane appears to be the trimeric form of sCAR. We often observed this when a large amount of protein was loaded. (c) ELISA binding assay suggesting sCAR-MTf can efficiently bind to Ad5 vectors. In the assay, Ad5 vectors were immobilized on the wells of a 96-well ELISA plate, and then incubated with different amount of sCAR or sCAR-MTf. The binding activity was analyzed by anti-His_6 antibody.

better than sCAR monomer. Two His_6 epitopes (in the middle and at the C-terminal end) were included to ensure protein purification. Similarly, a control protein sCAR that contains the sCAR, His_6, and fibritin trimerization domains, but not MTf domain was constructed. The expression of the adaptor proteins, which were driven by CMV promoter, was accomplished using Ad5 as gene delivery vector in mammalian HeLa cells as described in the Materials and methods. Proteins were purified from the media, as the adaptor proteins were designed as secreted proteins, and confirmed by Western blotting analysis using both anti-CAR and anti-MTf antibodies (Figure 1b).

Next, we examined whether the adaptor proteins retained their ability to interact with Ad5 vectors. *In vitro* ELISA (enzyme-linked immunoabsorbent assay) binding assay was performed in this regard. In the assay, unmodified Ad5 viral particles (VPs) were immobilized to each well of a 96-well ELISA plate, and incubated with different amount of fusion protein sCAR-MTf or control sCAR. After extensive wash, the binding activity was detected with anti-His_6 antibody, followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibody. The binding activity was assessed based on the AP activity. The results indicated that sCAR-MTf was capable of binding to Ad5 vectors, and the maximal binding could reach ~80% of that between sCAR and Ad5 vectors (Figure 1c).

Ad5 transcytosis mediated by sCAR-MTf

To test whether the bi-specific sCAR-MTf adaptor protein was able to re-direct Ad5 to MTf transcytosis pathway, we employed an *in vitro* BBB model system that has been widely used. The BBB model was established with BBMVEC cells in a transwell system, in which the BBMVEC cells were grown on the transwell inserts (polyester, 3.0 μm pore size, 12 mm diameter; Figure 2a). To establish a well-sealed endothelial barrier, the cells were cultured for 5–7 days following confluency, with media refreshed every other day. In the meantime, the transendothelial electrical resistance (TEER) was monitored. TEER reflects impedance of the passage of small ions through the physiological barrier, and has been widely used to measure BBB integrity.[23–25] Transcytosis assays were performed when the TEER of each BBB model reached the maximal value, which mostly were in the range of 100–150 Ω cm^2.

As previous studies have shown that MTf prefers apical-to-basal transcellular transport,[11] in the transcytosis assay, we pre-mixed Ad5 VPs with sCAR-MTf or control sCAR, and added the complexes to the upper chamber (apical side of the barrier). The cells were then

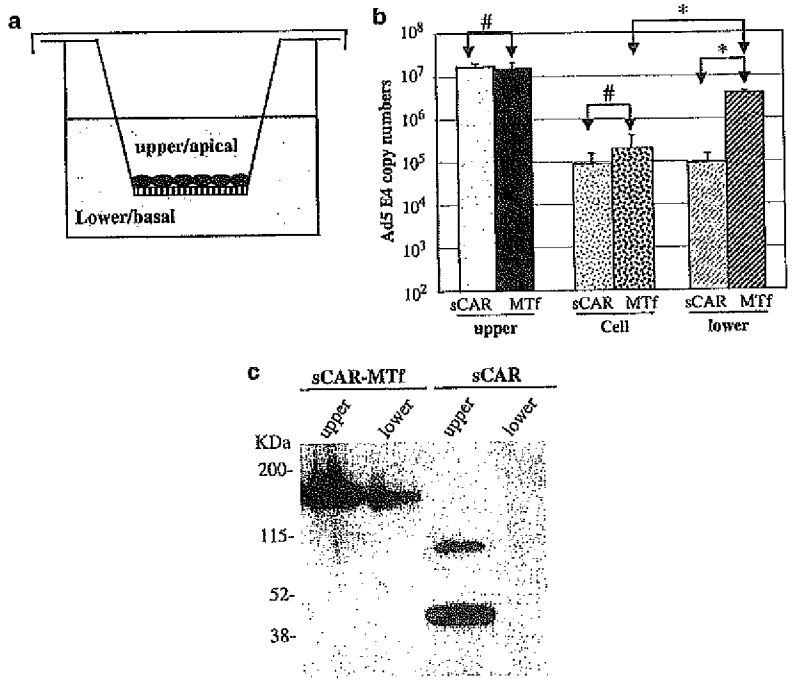

Figure 2 sCAR-MTf-mediated Ad5 transcytosis in the *in vitro* BBB model. (a) Diagram of the *in vitro* BBB model. BBMVEC cells were cultured on the transwell inserts. (b) Ad5 vectors were re-directed to transendothelial transport pathway in the presence of sCAR-MTf. Following transcytosis assay, samples were collected from both the apical and basal chambers. The BBMVEC cells were also collected by trypsin treatment. DNA isolation was performed for each sample and processed for quantitative PCR to detect Ad5 copy numbers using primers in the E4 region. Apical-to-basal transendothelial transport of Ad5 VPs were significantly increased in the presence of sCAR-MTf, suggesting Ad5 vectors were re-directed to transcytosis pathway by sCAR-MTf. *Indicates $P<0.01$, and #indicates $P>0.05$, as analyzed by Student's *t*-test. (c) Western blotting assay showing sCAR-MTf, but not sCAR, was transported to the basal chamber together with the Ad5 vectors, which further confirmed the transendothelial transport of the Ad5 vectors was mediated by sCAR-MTf.

incubated for 6 h in a 37°C, 5% CO_2-containing humidified incubator to allow transcytosis to occur. At the end of the experiments, we collected the samples from both the apical and basal chambers, and analyzed the presence of Ad5 virions and adaptor proteins. In addition, we also collected the BBMVEC cells by trypsin treatment to analyze the presence of Ad5 VPs that were endocytosed and remained inside the cells. Presence of the Ad5 particles was examined with quantitative real-time polymerase chain reaction (PCR) that measures the Ad5 E4 copy number. Western blotting assay was performed to detect the presence of the adaptor proteins after concentrating the samples using protein concentrator. As shown in Figure 2, we found that in the presence of sCAR-MTf, over 50 times more Ad5 particles, which accounted for about 5% of total Ad5 particles, were transported to the basal chambers compared to that in the presence of sCAR ($P<0.01$; Figure 2b). Consistent with this, we detected sCAR-MTf protein in the basal chamber samples, but not sCAR, although similar amounts of adaptor proteins were detected in the upper chambers, which indicated that similar amounts of the proteins were used in the assay (Figure 2c). Furthermore, our results showed that the amount of Ad5 particles that were endocytosed and remained inside the cells in the presence of sCAR-MTf was similar to that of in the presence of sCAR ($P=0.42$), and was significantly less than what was transcytosed to the basal chamber in the presence of sCAR-MTf ($P<0.01$) (Figure 2b). This suggests most Ad5 vectors were re-directed by sCAR-MTf to transcytosis pathway, not endocytosis pathway.

It should be noted that we have repeated the experiments many times, and often there were 40–50 times more transcytosed Ad5 particles in the presence of sCAR-MTf than that of sCAR, but the range could be as low as 10 times higher or as high as 100 times higher. The variation was probably due to variable cell culture/differentiation conditions and different batches of purified proteins. In addition, following the transcytosis assay, the TEER of each BBB model was checked and no significant difference from before the assay was found (Table 1), suggesting the BBB integrity was maintained during the transcytosis process. Together our data suggested the bi-specific adaptor protein sCAR-MTf was capable of re-directing Ad5 vectors to the transcytosis pathway.

Functionality of the transcytosed Ad5 VPs

We further investigated whether the transcytosed Ad5 VPs maintained their functional integrity during the transcytosis process. We evaluated the functionality of these Ad5 particles by examining their gene transfer efficacy. In the experiments, 50 μl of each sample collected from the basal chambers after transcytosis assay were used to infect a high-CAR cell line A549 cells, and their gene transfer efficacy was evaluated using the luciferase reporter gene that was incorporated in the E1 region of the Ad5 vectors. The actual multiplicity of infections (MOIs) were calculated based on the Ad5 copy numbers in the transcytosed samples, which were obtained by quantitative real-time PCR. Ad5 vectors that did not undergo transcytosis assay were used at various MOIs as control. Our results showed that the transcytosed Ad5 VPs retained their gene transfer efficacy (Figure 3), suggesting the transcytosis process did not harm the integrity of the Ad5 viruses.

Table 1 A representative set of TEER data before and after transcytosis assay

Group	TEER (Ω cm^2)		P-value
	Before transcytosis	*After transcytosis*	
Ad5+sCAR	124±8.5	113±12.2	0.27
Ad5+sCAR-MTf	130±15.0	112±4.7	0.13

Abbreviations: sCAR-MTf, soluble coxsackie-adenovirus receptor and the full-length melanotransferrin; TEER, transendothelial electrical resistance.
Three wells were used in the transcytosis assay for each group. Student's *t*-test was used to determine the *P*-value of the TEER before and after transcytosis. $P<0.05$ was considered statistically significant.

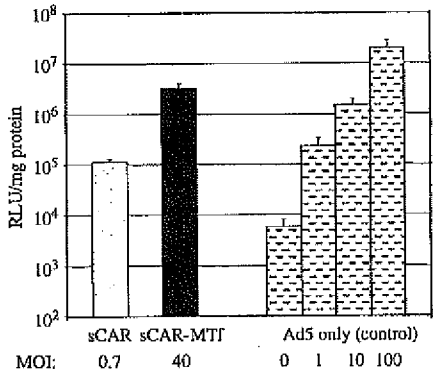

Figure 3 Infectivity of the transcytosed Ad5 VPs. Fifty microliters of each sample obtained from the basal chambers in the transcytosis assay were used to infect A549 cells. The actual MOIs of the infections as shown were calculated based on the corresponding quantitative PCR results. Freshly thawed Ad5 vectors that did not go through transcytosis assay were used to infect cells at MOI of 1, 10 and 100 as positive control, whereas uninfected cells (MOI = 0) as negative control. The relative light units per mg protein indicating the activity of the luciferase reporter that was incorporated in the Ad5 E1 region suggest the transcytosed Ad5 VPs maintained their infectivity/gene transfer efficacy.

Temperature-dependence of sCAR-MTf-mediated Ad5 transcytosis

Transcytosis is an active transcellular transport process. Temperature dependence is thus one characteristic of transcytosis. This temperature dependence was demonstrated for recombinant MTf transcytosis.[11] To confirm Ad5 vectors were indeed transported across the endothelial barrier via transcytosis pathway, we examined temperature dependence of this process by performing the transcytosis assay at either 4 or 37°C. As shown in Figure 4a, sCAR-MTf-mediated transcytosis was completely inhibited at 4°C ($P<0.01$), whereas in the control experiment with sCAR, there was no significant difference between 4 and 37°C ($P>0.05$).

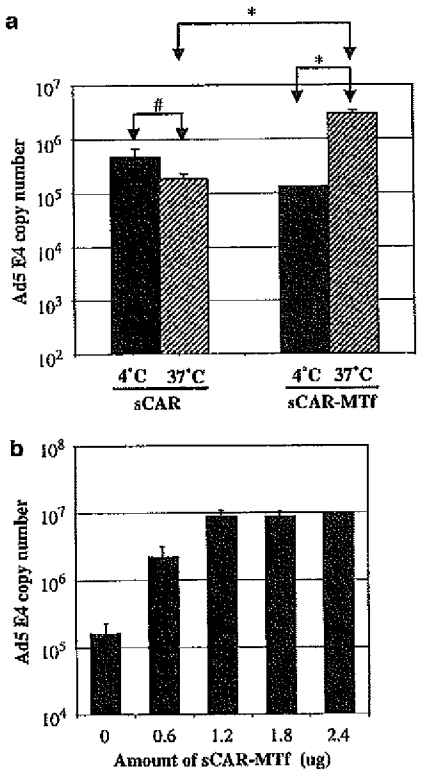

Figure 4 Characterization of the temperature- and dose dependence of sCAR-MTf-mediated Ad5 transcytosis. (a) The transcytosis assay was performed at either 4 or 37°C as described above. sCAR-MTf-mediated Ad5 transcytosis was completely inhibited at 4°C. *Indicates $P<0.01$, and #indicates $P>0.05$, as assessed by Student's *t*-test. (b) The transcytosis assay was performed with fixed amount of Ad5 (10^8 VPs) and various amount of sCAR-MTf protein at 37°C for 6 h as described above. Transendothelial transport of Ad5 was saturated when more than 1.2 μg of the adaptor protein were used.

Dose dependence of sCAR-MTf-mediated Ad5 transcytosis

MTf transcytosis is a receptor-mediated process, sCAR-MTf-mediated Ad5 transcytosis is thus expected to be dose dependent, and may be saturated. To test this and to determine the optimal dose for the transcytosis assay, we performed transcytosis assays using different amount of sCAR-MTf protein (Figure 4b). Fixed amount of Ad5 viruses (10^8 VPs) were used in each assay. After the transcytosis assay, the samples from the lower chambers were collected, viral DNA was isolated and subsequently used for quantitative PCR analysis. Our results suggested that efficacy of sCAR-MTf-mediated Ad5 transcellular transport was dependent on the dose of the adaptor protein, and the process was saturated in the presence of 1.2 μg sCAR-MTf adaptor protein when 10^8 Ad5 VPs were used (Figure 4b).

Directionality of sCAR-MTf-mediated Ad5 transcytosis

Recombinant MTf has been shown to prefer apical-to-basal transcytosis, although basal-to-apical could also occur.[11] To examine whether the sCAR-MTf adaptor protein maintained the apical-to-basal preference, which is essential for our purpose of directing Ad5 vectors into the brain, we performed transcytosis assay in both directions using the same amount of Ad5 vectors and the adaptor proteins. We did not detect significant basal-to-apical transcytosis of Ad5 vectors in the presence of sCAR-MTf, as the amount of transcytosed VPs was not significantly different from that of control experiment using sCAR, although sCAR-MTf-mediated Ad5 transcytosis occurred efficiently in apical-to-basal direction (Figure 5). These results suggest the adaptor protein has strong apical-to-basal preference in directing Ad5 vectors across the endothelial barrier, supporting its potential role of transporting Ad5 vectors towards the brain.

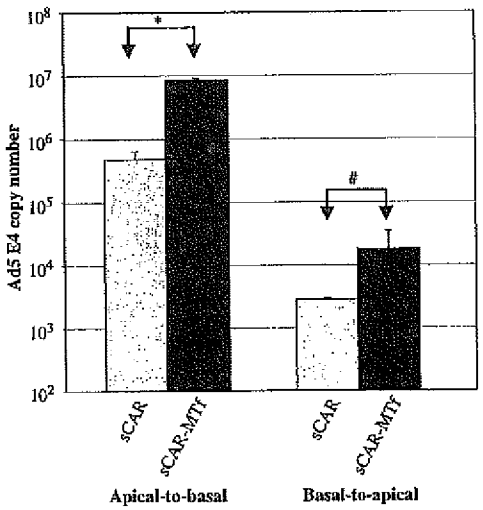

Figure 5 Directionality of sCAR-MTf-mediated Ad5 transcytosis. Ad5 (10^8 VPs) and sCAR-MTf protein (1.0 μg) were pre-mixed and added to either the apical or the basal chamber of each transwell containing the BBB model. Presence of Ad5 VPs in the opposite chamber was examined by quantitative PCR. *Indicates $P<0.01$, and #indicates $P>0.05$, as assessed by Student's *t*-test.

Discussion

In this study, we attempted to re-target Ad5 vectors to MTf transcytosis pathway, so that Ad5 vectors can traverse the BBB. We designed and constructed a bi-specific adaptor protein sCAR-MTf, and examined its ability to re-direct Ad5 across the BBB using an *in vitro* BBB model system established with BBMVEC cells. The adaptor protein was able to re-direct Ad5 vectors to traverse the BBB, often with an efficiency of 40–50 times higher than that of control. The Ad5 VPs undergone transcellular transport maintained their functionality/infectivity, as assessed by their gene transfer efficacy. In addition, the adaptor protein mediated Ad5 transcytosis was temperature- and dose dependent, which are the characteristics of receptor-mediated transcytosis, in accordance with previous studies.[11,12] Importantly, sCAR-MTf-mediated Ad5 transcytosis showed strong

apical-to-basal preference, arguing for its potential utility in transporting Ad5 vectors into brain tissue.

Employment of an *in vitro* model is essential to study whether the bi-specific adaptor protein sCAR-MTf was capable of re-directing Ad5 vectors traverse the BBB. In this work, the BBB model was established with BBMVEC cells that were obtained from Cell Applications Inc. (San Diego, CA, USA), at second passage (P2), and used at $P \leqslant 6$. The maximal TEER of each BBB model was often in the range of 100–150 Ω cm^2, which was reached around 5–7 days following confluency. There was no significant change in TEER when we cultured the BBB model up to 10 days after the cells became confluent. The TEER values of the BBB model in our study are comparable to other studies using BMVEC,[22,26] similar to rat BMVEC cells,[24] slightly lower than human BMVEC,[21] but higher than feline BMVEC.[23] Clearly, the TEER is different for different cell types. In addition, it has been shown that TEER can be modulated by many factors such as cytokines, cAMP, cGMP, nitric oxide, heat and co-culturing with other cells.[21,22,24–26]

Currently, no specific receptor has been identified for MTf. Although human MTf shares 39% homology with human serum transferrin,[13] TfR is found not to be responsible for MTf transcytosis. Instead, a member of LDL receptor family, LRP, may play an essential role in this regard.[11] In fact, LRP may be a common mediator for its binding partners to traverse the BBB. In addition to MTf, LDL, lactoferrin and LDL receptor-associated protein (RAP) have been found to cross the BBB with high efficiency and the receptor involved in their transcellular transport appears to be LRP.[11,12,27–30] *In vivo* transport across the BBB of these proteins may also help explain the observations that MTf, lactoferrin and LRP accumulate in the brain of patients with neurological diseases such as Alzheimer's disease.[31–34] Interestingly, LRP-mediated transcytosis may only be a feature of endothelial cells, as in other cell types or organs, majority of these proteins, once internalized, are found to be degraded or recycled.[35–37] Differentiation stage of endothelial cells also appears to play a role in determining what pathway the protein uptake is taken. For example, in growing brain capillary endothelial cells, LDL is classically internalized by the clathrin pathway, and directed to lysosomes for degradation. However, when the cells are fully differentiated, even though the classic degradation pathway (via lysosomes) is functional, LDL is mostly directed to non-degradation transcytosis pathway.[27] In accordance, our data showed that in the presence of sCAR-MTf, the majority of internalized Ad5 VPs were directed to the transcytosis pathway in the BBB model that is formed by differentiated cells, and very little remained inside the cells (Figure 2b). Apparently, the transcytosis pathway adopts a different trafficking mechanism from the classical endocytosis pathway, as it can bypass the lysosomal degradation. In this regard, caveolae, a type of vesicles that contain enriched caveolin and are non-clathrin coated, has been implicated in LRP-mediated LDL transcytosis through the brain microvascular endothelial cells.[27] The precise mechanism for transcytosis of the proteins across the BBB, however, remains to be investigated.

Adenovirus retargeting has been widely explored in terms of specific and effective gene delivery into certain target cells. It has been demonstrated that genetic incorporation of alternative targeting motifs into Ad5 fiber improves gene transfer efficacy and specificity.[38–41] However, genetic incorporation has a size limit. Epitopes less than 100 amino-acid residues may be incorporated into fiber knob domain without affecting viral assembly.[42] Alternatively, bi-specific adaptor proteins composed of sCAR and the alternate targeting motif such as epidermal growth factor, human transferrin and a single-chain antibody against carcinoembryonic antigen have been successfully employed to retarget Ad5 vectors.[17–19] *In vivo* stability of this strategy has also been demonstrated.[43,44] Our study adopted this adaptor strategy, as MTf is a large protein, and the domain responsible for its transcytosis is not defined thus far.

In our study, about 5% of Ad5 particles could be transcytosed across the BBB in the presence of sCAR-MTf. The percentage, however, may vary from 3 to 10% depending on cell condition and the batches of proteins. In addition, we observed about 0.1% Ad5 transendothelial transport in all of the negative control transcytosis assays, which include experiments with sCAR (Figures 2, 4a and 5) or Ad5 alone (Figure 4b). These apparently represented nonspecific Ad5 crossing the BBB, presumably owing to leakiness through the paracellular space or occasional escape of endosomes and exocytosis to the other side of the barrier.

Systemic gene delivery into brain is one of the most challenging problems faced by gene therapy investigators. The highly defensive BBB turns out to be the major hurdle. Engineering gene delivery vector so that it can traverse the BBB is thus a rational and attractive direction. In this study, we took advantage of the high efficiency of MTf transcytosis, and applied it in combination with Ad5 vector re-targeting strategy. Another potential advantage using sCAR-MTf for gene delivery into brain is that the major components of the adaptor protein, the extracellular domain of CAR and MTf, are endogenously expressed in human, therefore, the host immune response against the adaptor protein sCAR-MTf is expected to be minimal for *in vivo* applications. Although the data presented here were obtained *in vitro*, the proof-of-principle study appears to be very promising. *In vivo* utility of sCAR adaptor protein-mediated Ad5 retargeting strategy has been directly explored and demonstrated in an earlier study, in which an adaptor protein composed of sCAR and anti-carcinoembryonic antigen single chain antibody, sCAR-MFE, has been shown to re-direct Ad5 to the lungs of a transient transgenic mouse model overexpressing carcinoembryonic antigen in the pulmonary vasculature.[19] Our further efforts will be focused on investigation of the *in vivo* utility of sCAR-MTf-mediated Ad5 transcytosis, and the means of improving the efficiency of this strategy.

Materials and methods

Antibodies

The rabbit polyclonal antibody against CAR was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The mouse anti-His-Tag monoclonal antibody (Penta.His) was purchased from Qiagen (Valencia, CA, USA). To generate mouse monoclonal antibody against MTf, M-19 hybridoma cells that were raised against P97 antigen (i.e., MTf) were purchased

from American Type Culture Collection (ATCC). Antibody generation and purification were accomplished in the hybridoma core facility at University of Alabama at Birmingham. Secondary antibodies including AP-conjugated donkey anti-mouse and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Cells and cell culture

The human embryonic kidney cells transformed with Ad5-E1 DNA (293), the human lung carcinoma cell line A549, and the human melanoma cell line MeWo cells were purchased from ATCC. All of these cells were cultured in Dulbecco's modified Eagle medium-Ham's F12 medium that contains 10% fetal calf serum (FCS) and 2 mM L-glutamine, and grown in a 37°C, 5% CO_2 humidified incubator. The BBMVEC cells were purchased from Cell Applications Inc., and cultured in the complete BBMVEC growth media (also from Cell Applications Inc.). The cell culture flasks or transwell inserts were coated with attachment factor solution (Cell Applications Inc.) before cell seeding, as described in the manufacturer's protocol. The cells were maintained in a 37°C, 5% CO_2 humidified incubator, and the culture media were refreshed every other day until experimental execution.

Generation of Ad5 vector that expresses sCAR-MTf fusion protein

In order to express sCAR-MTf fusion protein using Ad5 vector, we first generated a shuttle vector containing the expression cassette, which is composed of (in order) CMV promoter, sCAR ectodomain that consists of its own leader sequence (amino-acid residues 1–236), a 5-amino-acid residue peptide link (GGPGS), a His_6 epitope in the middle, a bacteriophage T4 fibritin trimerization domain, full-length soluble MTf, and a C-terminal His_6 epitope. Construction of this cassette was carried out sequentially as described below. First, an extra His_6 epitope was cloned into the parent vector pcDNA3sCAR6hfibritin containing sCAR ectodomain, the peptide link (GGPGS), a His_6 epitope and T4 fibritin trimerization domain,[45] to generate pcDNA3sCAR6hf6h, in which the extra His_6 epitope was introduced into the C-terminal end of the expression cassette. The expression cassette then contained all components designed except the MTf fragment. This cassette including the CMV promoter and SV40pA was then amplified by PCR, and subcloned into pShuttle (Stratagene, La Jolla, CA, USA) vector, resulting in pShuttle.sCAR6hf6h.

Next, we obtained human MTf cDNA from human melanoma cell line MeWo cells. This was accomplished by reverse transcription (RT)-PCR. Briefly, the total RNA from MeWo cells was extracted using Qiagen RNeasy mini kit according to the manufacturer's manual. The RT reaction was then performed with universal primer oligo dT, and about 1 μg of total RNA was used as template. The full-length soluble form of MTf lacking the C-terminal GPI anchor (27 amino-acid residues) was subsequently amplified using the RT reaction as template. This MTf full-length fragment was then inserted into pShuttle.sCAR6hf6h in-frame at the position of between the fibritin trimerization domain and the C-terminal His_6 epitope. The resultant plasmid was named pShuttle.sCAR-MTf.

To incorporate the expression cassette of sCAR-MTf into the Ad5 vector, homologous recombination was performed between pShuttle.sCAR-MTf and Adeasy vector (Stratagene). This was accomplished by co-transformation of the linearized shuttle vector pShuttle.sCAR-MTf and backbone pAdeasy into *Escherichia coli* BJ5183. The recombinants were initially screened by DNA isolation and restriction digestions. The plasmid DNA of positive candidates were then transformed into bacteria DH10B, and more DNA was isolated and screened. The final positive recombinants were confirmed by sequencing analysis, and the resultant Ad5 vector was named pAdeasy.sCAR-MTf.

To rescue the viruses encoding sCAR-MTf, pAdeasy.sCAR-MTf was digested with Pac I, and transfected into the Ad-E1 expressing 293 cells with Superfect (Qiagen). After plaques were formed, they were collected and processed for large-scale amplification in 293 cells. The viruses (named Ad5.sCAR-MTf) were then purified by standard CsCl gradient centrifugation.[46]

Protein expression and purification

We employed HeLa cells to express and purify the adaptor protein. Twenty flasks (185 cm^2) of HeLa cells that were grown to 80% confluency were infected with the Ad5 viruses encoding the adaptor protein at MOI of 500. The infected cells were then cultured for 48 h in a 37°C, 5% CO_2 humidified incubator before protein purification. As the adaptor protein was designed as secreted protein, we collected the culture media for protein purification. Purification of the protein was performed based on the His_6 epitopes that were incorporated in the adaptor protein, and the Qiaexpressionist™ system (Qiagen) was used to purify the His_6-tagged protein. For purification, we first concentrated the media to ~50 ml using protein concentrator columns (10- or 50-kDa MWCO, Millipore, Billerica, MA, USA) according to the manufacturer's manual. Then, 1/10 volume of 10 × supplemental buffer (500 mM NaH_2PO_4, pH 8.0, 1.5 M NaCl, 100 mM imidazole) was added to the media to adjust salt and pH condition of the sample. Next, 1 ml of Ni-NTA agarose (Qiagen) that was prewashed with phosphate-buffered saline (PBS) was added into the media and the sample was incubated at 4°C for 2 h on an end-over-end shaker. The Ni-NTA agarose beads were then collected by centrifuging the sample at 1000 r.p.m. for 5 min. After washing the beads twice with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween-20, pH. 8.0), the adaptor protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween-20, pH. 8.0). The protein concentration was determined using Bio-Rad *DC* protein assay kit.

Enzyme-linked immunoabsorbent assay

In vitro ELISA binding assay was performed similarly as described previously.[39] In brief, 10^9 VPs of Ad5 was immobilized on wells of a 96-well ELISA plate (Maxisorp; Nunc, Roskilde, Denmark) by overnight incubation at 4°C. The wells were then washed four times with Tris-buffered saline (TBS) containing 0.05% Tween 20, blocked with blocking solution (2% bovine serum albumin (BSA)+0.05% Tween 20 in TBS) for 1 h, and

incubated with different concentrations of purified adaptor proteins in binding buffer (0.5% BSA+0.05% Tween 20 in TBS) overnight at 4°C. The binding of adaptor protein to Ad5 viruses was detected by incubating with anti-His tag antibody and AP-conjugated corresponding secondary antibody, followed by color reaction that detects AP activity. The color reaction was performed using *p*-nitrophenyl phosphate (Sigma, St Louis, MO, USA) as recommended by the manufacturer, and absorbance at 405 nm (OD_{405}) was obtained using a microplate reader (Molecular Devices, Menlo Park, CA, USA).

Western blotting assay

In the assay, protein samples were boiled in Laemmli sample buffer, separated on 4–15% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was then incubated with blocking solution containing 5% skim milk and 0.05% Tween 20 in TBS for 1 h at room temperature and processed for incubation with primary antibody against CAR or MTf. After washing with TBS and re-blocking, the membrane was incubated with HRP-conjugated secondary antibody for 2 h at room temperature. After extensive washing, the immunoreactive bands were detected by enhanced chemiluminescence plus Western blotting detection system as recommended by the manufacturer (Amersham Biosciences, Piscataway, NJ, USA).

In vitro *BBB model and transcytosis assay*

The *in vitro* BBB model was established by culturing BBMVEC cells in a transwell system, in which the BBMVEC cells were grown on the transwell inserts (polyester, 3.0 μm pore size and 12 mm diameter, Costar, Corning Incorporated, Acton, MA, USA). To establish a well-sealed endothelial barrier, the cells were continued culturing for 5–7 days after confluency, with media refreshed every other day. In the meantime, the TEER was monitored using millicell-ERS apparatus (Millipore). As the TEER is inversely proportional to the area of the tissue, the standard practice is to report TEER as the product of the resistance (Ω) and the growth area (cm^2). In this study, the TEER of each BBB was obtained by subtracting the background resistance (inserts without cells) from the measured barrier resistance, then multiplying by the growth area of 12-mm inserts (1.13 cm^2). Transcytosis assay was performed when TEER reached 100–150 Ω cm^2, the maximal TEER the BBB models could reach in our study.

The transcytosis assay was performed as following, which was modified from previous studies.[11,18] In the assay, the BBMVEC cells grown in the transwell system were pre-incubated in Ringer-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) solution for 2 h at 37°C, 5% CO_2. The virus–adaptor protein complex was formed by mixing 10^8 VPs of Ad5 with 1 μg of adaptor proteins in 250 μl of Ringer-HEPES solution and incubating at 37°C for 15 min. The virus–protein complex was then added to the apical chamber of each well containing the BBB model, in which the basal chamber contains 700 μl of Ringer-HEPES solution. The cells were incubated again in a 37°C, 5% CO_2 humidified incubator for 6 h. At the end of the experiments, the samples from basal chambers were collected to analyze transcytosed VPs and proteins. Samples from the apical chambers and cell layers were also collected and analyzed.

To analyze the presence of VPs, 200 μl of the samples were used to extract DNA, and processed for real-time quantitative PCR that assessed the Ad5 E4 copy number. For Western blotting assay, the samples were concentrated to about 100 μl with protein concentrator (10-kDa MWCO, Sartorius, Vivascience, Edgewood, NY, USA), then processed for Western blotting assay as described above.

Gene transfer assay

Gene transfer efficacy of the transcytosed Ad5 vectors was assessed in A549 cells by measuring luciferase activity, essentially as described previously.[46] In brief, 50 μl of each sample from the basal chambers after transcytosis assay were diluted in 100 μl culture media containing 2% FCS, and used to infect A549 cells plated in a 48-well cell culture plate. Two hours later, 150 μl of complete culture media containing 10% FCS were added into each well, and the cells were continued in culture for 24 h in a 37°C, 5% CO_2 humidified incubator. To measure the luciferase activity, the cells were washed with PBS, and lysed by one freeze-thaw cycle in 100 μl of reporter lysis buffer (Promega, Madison, WI, USA). Ten microliters of each sample were used to measure the luciferase activity using a luciferase assay kit (Promega) and a luminometer (Berthold, Gaithersburg, MD, USA). The total amount of protein in each sample was determined by Bro-Rad *DC* protein Assay kit (Bio-Rad).

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A Unique Carrier for Delivery of Therapeutic Compounds beyond the Blood-Brain Barrier

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Abstract

Background: Therapeutic intervention in many neurological diseases is thwarted by the physical obstacle formed by the blood-brain barrier (BBB) that excludes most drugs from entering the brain from the blood. Thus, identifying efficacious modes of drug delivery to the brain remains a "holy grail" in molecular medicine and nanobiotechnology. Brain capillaries, that comprise the BBB, possess an endogenous receptor that ferries an iron-transport protein, termed p97 (melanotransferrin), across the BBB. Here, we explored the hypothesis that therapeutic drugs "piggybacked" as conjugates of p97 can be shuttled across the BBB for treatment of otherwise inoperable brain tumors.

Approach: Human p97 was covalently linked with the chemotherapeutic agents paclitaxel (PTAX) or adriamycin (ADR) and following intravenous injection, measured their penetration into brain tissue and other organs using radiolabeled and fluorescent derivatives of the drugs. In order to establish efficacy of the conjugates, we used nude mouse models to assess p97-drug conjugate activity towards glioma and mammary tumors growing subcutaneously compared to those growing intracranially.

Principal Findings: Bolus-injected p97-drug conjugates and unconjugated p97 traversed brain capillary endothelium within a few minutes and accumulated to 1–2% of the injected by 24 hours. Brain delivery with p97-drug conjugates was quantitatively 10 fold higher than with free drug controls. Furthermore, both free-ADR and p97-ADR conjugates equally inhibited the subcutaneous growth of gliomas growing outside the brain. Evocatively, only p97-ADR conjugates significantly prolonged the survival of animals bearing intracranial gliomas or mammary tumors when compared to similar cumulated doses of free-ADR.

Significance: This study provides the initial proof of concept for p97 as a carrier capable of shuttling therapeutic levels of drugs from the blood to the brain for the treatment of neurological disorders, including classes of resident and metastatic brain tumors. It may be prudent, therefore, to consider implementation of this novel delivery platform in various clinical settings for therapeutic intervention in acute and chronic neurological diseases.

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Competing Interests: Since submission, patents held by the authors and the University of British Columbia that are associated with the technologies described herein have been acquired by bIOasis Technologies Inc.

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Introduction

Neurological diseases associated with cancers, inborn errors of metabolism (such as lysosomal storage diseases), infectious diseases and aging create a significant social and economic burden. Demographics predict that, in the aging populations of the economically developed world, the incidence of cardiovascular disease, dementias and cancer will continue to dramatically

increase over the next 20 years. Despite advances in molecular screening technologies, which have spawned new drug targets and therapeutic candidates for neurological diseases, these technologies have yet to find conduits for successful clinical application due in part to disease complexity, the individual variability within the human population and the poor early diagnosis of disease. However, the most serious impediment to pharmaceutical treatment is inefficient delivery of drugs to the disease-affected brain tissue, due to limitations and restrictions dictated by the existence of the blood-brain barrier (BBB) [1]. Designing efficient 'vectors' (antibodies, protein carriers, viruses, nanoparticles) to navigate and deliver therapeutics across the BBB in a controlled and non-invasive manner remains one of the key goals of drug development for brain diseases.

The BBB is found in all vertebrates and is selectively permeable. The BBB endothelial cells are known as the "gatekeepers of the brain" and the barrier is formed by the presence of high resistance tight junctions that fuse brain capillary endothelia into a continuous tubular cell layer separating blood from the brain. In addition to keeping unwanted substances out, the BBB helps retain brain-synthesized compounds, such as neurotransmitters. Fine structural differences exist between the endothelia of the brain capillaries and endothelia in other capillaries. These include tight junctions between adjacent endothelial cells [2], a paucity of pinocytotic vesicles [3,4] and a lack of fenestrations (perforations) [5–7]. The cerebral endothelium forms tighter junctions than other endothelia that are characterized by greater electrical resistance and contain specific proteins, including enzymes and transporters, whose expression appears to be augmented in comparison to other endothelia [4,8–11]. The physiochemical properties of the penetrating substance largely determine whether or not it can penetrate or be transported across the BBB. In general, small hydrophobic solutes can readily cross the BBB, while hydrophilic substances are selectively transported across the barrier by specific trans-endothelial membrane carrier proteins. Thus, many substances in the blood cannot transit the BBB since they are not compatible with the resident carrier systems.

The tightly sealed brain vasculature forming the BBB precludes virtually all systemically injected macromolecular drug compounds and most hydrophilic drugs from entering the brain. Furthermore, efflux transporter proteins expressed at the BBB, such as P-glycoprotein (Pgp1) [1], present significant problems for the treatment of brain tumors with current chemotherapeutics [12] as they act to pump small hydrophobic chemotherapeutic agents out of the brain. Many methods developed to enhance the delivery of drugs to treat brain tumors have failed to provide significant improvements to long-term survival [13–25]. Radical methods to transiently increase the permeability of the BBB allowing diffusion of injected drugs into the brain [26] [27] [28] cause damage by uncontrolled entry of the blood constituents into the brain. Thus, specific drug design is limited by factors such as lipid solubility, charge, molecular weight and the antiport action of specific transporters [29,30]. A large assortment of drugs conjugated to peptides [26,31–33], to proteins [1,31,32], or to antibodies [28,34,35] able to bind to receptors expressed on the luminal surface of the BBB have been investigated. MRC OX26, a monoclonal antibody against the rat transferrin (Tf) receptor [36,37] has been used to study transport across the BBB [35,38,39]. Although partially effective for transport into the brain, the use of antibodies such as MRC OX26 appears to be limited due to saturation of the receptor with antibody, low dissociation rate of the antibody (and indirectly its potential payload of therapeutic compounds), and recycling of the receptor back to the blood [40]. Furthermore, hypersensitivity resulting in hyperimmunity against the foreign monoclonal antibody carrier may also limit repeated treatments with the antibody-drug conjugates. In addition, many targeted receptors are widely expressed in other tissues resulting in potential toxicity [36,37,41]. Finally, lost in the considerable data on potential transporters, carriers and delivery systems it is an unfortunate realization that, at present, none of these approaches are efficacious in treating diseases that lay beyond the BBB. Thus, novel approaches are required to increase the survival of patients with acute and chronic neurological diseases.

Here we have focused on the iron binding protein p97 (melanotransferrin), a protein closely related to Tf and lactoferrin (Lf) [42]. As a result of alternative splicing, p97 exists in both a soluble form and a cell surface GPI-linked form [43]. However, in normal brain it appears to discretely localize on the surface of endothelial cells and transiting through brain capillary endothelium [10,44]. Studies on the structure and function of p97 suggest it might be an ideal carrier for transport of drug conjugates into the brain [31,33,34,37,45,46]. Recombinant p97 is actively transported across the BBB in an *in-vitro* model [47] of BBB transcytosis [48], with a transport rate 10 to 15 times higher than that of either Tf or Lf. [48]. In addition, studies on its biodistribution support the concept that p97 injected intravenously preferentially distributes in brain tissue [39,49]. Furthermore, the mechanism of transport is likely receptor-mediated transcytosis, possibly involving a member of the low-density lipoprotein receptor–related protein family (LRP) [49–52]. The p97 protein exists at low serum concentrations (under 7.5 ng/mL = 0.08 nM in healthy adults) suggesting that the native protein should not significantly block the binding of exogenously-injected p97 conjugates from reaching the receptors in the BBB. Furthermore, the transcytosis of p97 is likely to allow piggybacked therapeutic compounds to bypass the efflux transporter Pgp-1. Thus, based on these collective properties, we hypothesized that p97 may be an attractive new candidate as a drug delivery vector. Here we test this hypothesis in a mouse model by assessing the chemotherapeutic activity of p97-drug conjugates within the brain in comparison with unconjugated drugs. We demonstrate that intervention with p97-drug conjugates provides a marked improvement over current ineffective chemotherapies for cancers of the brain. The p97 protein, therefore, is the first carrier that efficiently transports chemotherapeutic agents across the BBB that therapeutically modulates disease within the brain. Thus, p97 may have wide utility as a drug delivery vehicle for the treatment of a variety of inoperable neurological conditions.

Methods

Cells and Animals

C6 rat glioma (ATCC CRL-2199) and ZR-75-1 human mammary tumor (ATCC CRL-1500) cells were cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2 in air atmosphere. BHK TK$^-$ ts13 cells transfected with a full-length human p97 cDNA [53] were cultured in DMEM supplemented with 10% FBS, 20 mM HEPES, 2 mM L-glutamine, 0.08 mM zinc sulphate and 500 μM methotrexate [53]. Soluble p97 was affinity purified as previously described [53], its concentration determined a quantitative antibody sandwich assay [54] and its purity determined by SDS-PAGE analysis [54]. Female NSWNU (m) Swiss nu/nu mice aged 6–8 weeks were used for tumor models. Both male and female C57Bl/6 mice, aged 6 to 8 weeks, were used in all other studies. All procedures involving mice were in accordance with guidelines set by the UBC Animal Care Committee, which states that mice

must be euthanized if they lose more than 20% of their starting weight or if the tumor size should exceed 5% of the animal's normal weight.

Microscopy

Holo-human p97, holo-human transferrin (huTf), and holo-murine transferrin, mTf, (Sigma) were labeled with Alexa Fluor 488 (Alexa 488) protein labeling kit (Molecular Probes, MP) for confocal microscopy or using a DIG protein labeling kit (Boehringer-Mannheim) for electron microscopy (EM). Labeled or unlabeled holo-proteins (0.3 mg) were injected into the tail vein. Human Tf was used in order to allow differentiation from endogenous murine Tf. After 1 h, mice were perfused through the left ventricle with PBS, followed by 4% paraformaldehyde in PBS. The brains were immediately dissected, paraffin embedded and later sectioned. Brains were stained with either the anti-human p97 monoclonal antibody, L235 [55] or the rabbit anti-human Tf antibody (Research Diagnostics). Antibody binding to these tissue sections was subsequently visualized with goat anti-mouse Ig Alexa 488 or goat anti-rabbit Ig Alexa 488 (Molecular Probes).

Fluorescent PTAX (Oregon Green® 488 paclitaxel; Molecular Probes) was diluted in buffer and 100 µg were injected *i.v.* into each mouse. In addition, Oregon Green® 488 was conjugated to p97 as directed by the supplier, and 0.2 ml of the conjugate containing 100 µg of the dye was injected *i.v.* Injection was repeated 3 times during the day and mice were sacrificed by the end of the day. Then organs were harvested and analyzed later by fluorescent microscopy.

For electron microscopy (EM), 0.4 mg of p97 and p97-DIG labeled with 13 nm and 5 nm gold particles (British Biocell) respectively, were injected simultaneously into the tail vein. After 1 hr, mice were perfused and brains processed for EM [39]. Thin sections were stained with anti-DIG antibody (British Biocell) or visualized by gold enhancement (Nanoprobes).

Stability of ^{125}I-p97 in Serum and Urine

Urine and serum samples were centrifuged at 9000× g for 15 min at 4°C. Samples were reconstituted in SDS-PAGE buffer containing a final concentration of 1% SDS and were heated to 95°C for 10 min before gel separation. For reducing conditions, 2% ß-mercaptoethanol was included in the SDS-PAGE buffer. SDS PAGE gels (12%) were run with Tris-glycine-SDS buffer and after electrophoresis were dried and exposed to Kodak XAR Film for 2–4 days.

Uptake of ^{125}I-p97 into the Brain

To measure the brain uptake of ^{125}I-p97, mice were each given approximately 4 pmol of ^{125}I-p97 (0.026 µg in 1.8 µCi) in 200 µl of injection solution 14.2 µg/mouse lactated Ringer's solution or 0.25 M phoshate buffered saline (PBS) containing 1% bovine serum albumin (BSA) through the tail vein at time 0 with a Hamilton syringe. After the radiolabeled p97 has been circulating in the blood for 2 hours, organs were collected for radioactivity measurement. The serum and brain samples were collected and the levels of radioactivity were measured.

Multiple Regression Analysis of Entry of ^{125}I-p97 into the Brain

^{99}Tc-albumin with comparable radioactivity was included in this injection solution as a vascular marker. At 5, 10, 15, 20, 25 and 30 min after injection, blood was collected from the right carotid artery and the mice ($n=5$ for each time point) were immediately decapitated. The radioactivity in 50 µl of serum and of weighed brain samples were measured by a dual channel γ-counter. This study provided information on the brain uptake of ^{125}I-p97 at certain time points and further served as the control for the concurrent investigation into whether the brain entry of ^{125}I-p97 was saturable. Self-inhibition of ^{125}I-p97 brain uptake was tested by inclusion of unlabeled p97 (1:500 and 1:1000) in the injection mixture (7.1 mg/mouse; $n=3$ for each time point). Distribution of albumin in different organs was measured in similar fashion as the p97. To correct for the decrease of ^{125}I-p97 concentration from blood with time, exposure time (t), which his the integral serum radioactivity at time 0 to time t divided by the serum radioactivity at time t [56,57], was calculated. The organ/serum ratio of radioactivity (ml/g) was plotted against exposure time. The slope of the linear part of this regression line represents the influx rate, and the intercept at time 0 is the initial volume of distribution of ^{125}I-p97 in the organs for each group. The half-time disappearance was determined from the regression line obtained from the plot of the logarithm of brain radioactivity against time. The unidirectional influx constant (K_i), expressed in µl/g-min, and the apparent volume of distribution of the brain (V_i), in µl/g were determined from the linear portion of the relationship between brain/serum ratios and t with the equation; brain/serum ratio [(cpm/g brain)/cpm/µl/g serum)] = K_i (t)+V_i.

Capillary Depletion Method to Determine the Brain Compartmental Distribution of ^{125}I-p97

A capillary depletion procedure [58,59] was employed to separate the cerebral capillaries from the brain parenchyma. Blood was collected from the carotid aorta of mice ($n=5$) at 60 min following the *i.v.* tail injection of 3.6 mCi ^{125}I-p97. Subsequently, the jugular veins were cut, the descending aorta was blocked and the mice received an intracardial perfusion of injection solution before decapitation. For each mouse, the brain was dissected, weighed, homogenized by three passages through a 20 gauge syringe in 2 ml of 1 mg/ml collagenase/dispase. The mixture was incubated at 37°C for 30 to 40 min and then washed one time in PBS. The material was resuspended in 6 ml PBS and equally split into 3 Corex tubes. Two ml of 26% dextran was added to each tube to enable pelleting of the brain capillaries. The samples were mixed and then centrifuged at 5400 *g* for 15 min at 4°C. The resulting pellet (cerebral capillaries component) and the supernatant (brain parenchymal/interstitial fluid space) were carefully separated and the percent contamination of the supernatant with vasculature was assessed by measuring the specific activity of the endothelial marker, γ-glutamyl transpeptidase. Radioactivity of each sample was counted with a dual channel γ-counter. The ratios of radioactivity of ^{125}I-p97 in the supernatant (parenchyma) or pellet (capillary) over serum were calculated, and the contamination of the vascular component was further corrected by subtraction of the ^{99}Tc-albumin ratios.

Synthesis and Analysis of p97-PTAX Conjugates

Conjugation of PTAX to p97 followed the procedures described previously [60]. The MSR of p97 was determined by absorbance analysis and was determined to be similar to transferrin, which has a conjugate to carrier ratio of 5 PTAX molecules per p97 molecule [60]. The stability of the conjugates in buffer and in mouse sera was measured by HPLC at room temperature (RT) over a period of 1 to 120 hrs.

Extraction Method for PTAX analysis

Tissue extraction was performed by a modification of the method of Sparreboom *et al.*[61] and the PTAX metabolites were identified

according to the method of Royer *et al.*[62]. Thawed tissue was Dounce homogenized, then pipetted into a glass tube and made up to 1 ml with 4% BSA solution. Tissue samples underwent diethyl ether extraction and solid-phase extraction (SPE). Plasma samples consisted of 250 µl of thawed murine plasma and underwent SPE only. Samples with less than 250 µl volume were filled up with human citrate-phosphate-dextrose/plasma. To each sample 10 µl of 50 µM docetaxel solution (Taxotere diluted with absolute ethanol; Rhone-Poulenc Rorer) were added as internal standard. HPLC analysis was performed on an Agilent HPLC apparatus. A stainless steel (125×4 mm) analytical column equipped with a guard column (4×4 mm), both packed with 5 µm LiChrospher 100 RP-18 material, was cooled to 33°C. The mobile phase consisted of acetonitrile/methanol/0.2 M ammonium acetate buffer, pH 5.0, 38:10.5:51.5 (vol/vol). UV detection was performed at 227 nm, and washing gradient was applied after each run (additional 35% vol/vol acetonitrile). Injection volumes were either 50 or 100 µl. For calibration, PTAX and docetaxel stock solutions in anhydrous ethanol, stored at −20°C, were diluted with mobile phase (1:100) on the day of analysis. The method of analysis was validated for murine plasma, brain, liver, and kidney samples. Correlation coefficients for PTAX calibration curves in the concentration range 50–1000 nM were greater than 0.999. Recovery rates for PTAX ranged from 70 to 85%, depending on PTAX concentration and the type of tissue.

Brain Uptake Trials Involving p97-PTAX

Delivery of PTAX to brain was examined by the following procedure: mice (n = 10) received 5 injections of 8 mg/kg of PTAX over one week. Before organ dissection, mice were anesthetized (ketamine-HCl 100 mg/kg, xylazine 10 mg/kg) and a 300 µL sample of blood removed by cardiac puncture. The left atrium was snipped with scissors and the mouse was perfused with heparinized saline from a peristaltic pump through a 27 gauge needle, thereby removing all blood. PTAX concentration was measured in brain and serum. In 5 additional mice, delivery and stability of PTAX was measured after a single injection and the blood concentration and stability of the conjugate (p97-PTAX) was measured at different time points (0.25, 0.50, 1, 1.5, 2, 6 and 12 hrs; refer to Table 1) based on the free amount of PTAX present in serum.

Synthesis and Analysis of p97-ADR Conjugates

Conjugation of ADR [(8S,10S)-10-(4-amino-5-hydroxy-6-methyl-tetrahydro-2H-pyran-2-yloxy)-6,8,11-trihydroxy-8-(2-hydroxy-acetyl)-1-methoxy-7,8,9,10 tetrahydrotetracene-5,12-dione] to p97 was achieved by cross-linking p97-N-succinimidyl S-acetylthioacetate (p97-SATA) and ADR-succinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (ADR-SMCC) derivatives. SATA (Pierce Chemical Co, St. Louis MO) and the p97-SATA were prepared according to instructions supplied by the supplier. Activated ADR-SMCC (>96% pure) was synthesized by Albany Molecular Research Inc. The conjugation of deacylated p97-SATA to the activated ADR-SMCC was achieved by combining these compounds at 4°C overnight. Conjugates (p97-ADR) were purified on 5 mL D-Salt Excellulose (Pierce Chemical Co.) desalting columns equilibrated with PBS. Purified p97-ADR was assessed by SDS-PAGE, anion exchange chromatography and Western blot analysis using the primary anti-human p97 monoclonal mouse antibody L235 [63]. Individual conjugates were designated as SYN002, SYN018, SYN019, and SYN020. The molecular substitution ratio (MSR) or moles of ADR bound to moles of p97, were determined by absorbance analysis from standard curves established for ADR and p97 at 486 and 280 nm wavelength. The two conjugates were found to have 4 to 7 molecules of ADR per molecule of p97 at an ADR concentration of 17 to 60.6 µg/ml.

Radiolabeling of Compounds

p97, Lf, and p97-ADR conjugates were iodinated using a standard chloramine T protocol [52]. The specific activity was calculated from the radioactivity of the precipitable fraction after trichloroacetic acid (TCA) precipitation. Bovine serum albumin (BSA) in the form of ^{99}Tc-albumin was purchased from Amersham (now GE Healthcare) and had a specific activity of 100 Ci/g. [^{14}C]-labeled ADR was purchased from Nycomed.

Stability and Quality of p97-ADR Conjugates

To determine the stability of the p97-ADR conjugate, SYN002 was prepared with [^{14}C]-labeled ADR. Aliquots of 200 µL were added to 1 mL of mouse sera and incubated at 37°C. Over a period of 18 hr, 25 µL aliquots were removed and the CPM of the TCA-precipitable fraction was determined. The samples were run on 12% SDS-PAGE gels as described above and protein positions were visualized after exposure to Kodak XAR film for 25 days.

Treatment of Mice Bearing Subcutaneous Tumors

C6 glioma tumors were established subcutaneously in mice by injecting 50 µL of 1×10^5 rat C6 glioma cells into the right flank. Groups of 3–10 mice were treated (detailed in Table 2), starting shortly after the tumor cells, with p97-ADR conjugate (SYN002 -

Table 1. Concentration and Stability of p97-PTAX *in vitro* and *in vivo*.

Concentration of p97-PTAX in Buffer (ng/ml)		Concentration of p97-PTAX in Serum After injection (nmol/kg)		Concentration of p97-PTAX in Brain After Injection (nmol/kg)	
1 hr	80±2.1	15 min	2.0±1.37	15 min	0.06±0.001
5 hrs	100±9.1	30 min	0.6±0.14	30 min	0.007±0.001
24 hrs	120±8.4	1 h	1.0±0.8	1 h	0.01±0.003
48 hrs	100±7.8	1.5 h	1.5±0.2	1.5 h	0.015±0.0002
72 hrs	80±2.4	2 h	2.0±0.2	2 h	0.01±0.004
94 hrs	30±4.6	6 hrs	0.8±0.42	6 hrs	0.09±0.006
120 hrs	10±5.8	12 hrs	0.2±0.01	12 hrs	0.07±0.003

Concentration of p97-PTAX conjugate in buffer at RT, in serum after *i.v.* injections and in mouse brain after *i.v.* injection. Free PTAX could not be detected in brain. Sample number in buffer and serum, $n=5$; in brain, $n=10$.
+/− are standard deviations.
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Table 2. Injection Schedule of ADR-conjugates in Different Mouse Models.

Trial	n	Tumour Cell type	Tumour location	Treatment	Injection schedule (Day)	Total ADR (mg/kg)
1	9	C6	Subcutaneous	PBS	3,4,5,6,10,11,12,13 (8)	0
	9	C6	Subcutaneous	ADR	3,4,5,6,10,11,12,13 (8)	4
	9	C6	Subcutaneous	SYN002	3,4,5,6,10,11,12,13 (8)	4
2	10	ZR-75-1	Intracranial	PBS	3,4,5,6,7,10,11,12,13,14 (10)	0
	10	ZR-75-1	Intracranial	ADR	3,4,5,6,7,10,11,12,13,14 (10)	20
	10	ZR-75-1	Intracranial	SYN002	3,4,5,6,7,10,11,12,13,14 (10)	5.5
3	10	C6	Intracranial	PBS	1,3,7,10,14 (5)	0
	10	C6	Intracranial	SYN018	1,3,7,10,14 (5)	0.49
4	10	C6	Intracranial	PBS	2,9,17,20,25 (5)	0
	10	C6	Intracranial	ADR	2,9,17,20,25 (5)	20
	10	C6	Intracranial	SYN002	2,9,17,20,25 (5)	2.75
5	2	N/A	N/A	BSA	(1)	0
	2	N/A	N/A	Lf	(1)	0
	5	N/A	N/A	p97	(1)	0
	3	N/A	N/A	SYN019	(1)	0.1
	3	N/A	N/A	SYN020	(1)	0.1

Injection schedule indicates the number days after the tumor implant when the single injections of the specific treatments were administered. The number in the brackets indicates the total number of injections. All injections were *i.v.* made via the tail vein with the exception of Trial 4, which used intra-jugular injections.
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0.0625 mg/mL ADR), ADR (0.0625 mg/mL in PBS) or PBS alone by 5–10 tail vein injections (8 μL/g body mass each time) over a period of 14–25 days for a total free or conjugated ADR at 4 mg/kg. Tumor size was monitored over 19 days. At the end of the trial, serum was collected and analyzed for two markers of cardiotoxicity: creatine kinase (CPK) and lactate dehydrogenase (LDH)[64,65]. Control treatments were either PBS or ADR alone.

Treatment of Mice Bearing Intracranial Tumors

Mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). A motorized injector delivered 4×10^5 C6 glioma or 1×10^6 ZR-75-1 cells in 5 μL PBS at rate of 1 μL/min from a 25 μL Hamilton syringe through a 27 gauge needle to a position located 3 mm below the surface of the skull, 3 mm in front of coronal suture and 3 mm to the right of midline. The needle was removed slowly two minutes after the completion of the injection. The site was wiped with an alcohol soaked swab and sealed using sterile bone wax, and the scalp incision closed with sterile clips. Mice bearing intracranial C6 glioma were treated with repeated injections (8 μL/g body mass - jugular vein) of p97-ADR (SYN002 - 0.06875 mg/ml ADR) for a total dose of 5.5 mg/kg ADR, or free ADR (0.5 mg/ml ADR in PBS) for a total dose of 20 mg/kg, or PBS alone.

In Trial 2, the effectiveness of SYN002 conjugate against intracranial ZR-5-1 mammary tumors was assessed by repeated tail vein injections of 8 μL per g body weight solutions containing PBS, ADR (0.25 mg/ml ADR in PBS) or SYN002 (0.06875 mg/ml ADR). The dosing regime is outlined in Table 2 and carried out over a period of 15 days to a total of 20 mg/kg free ADR or 5.5 mg/kg conjugated ADR. Survival was reported as the percentage of total mice receiving an intracranial tumor. The data were represented as Kaplan-Meier survival curves. Mean and median survival in days since tumor implantation were calculated for each treatment. Efficacy of the conjugate and free ADR were determined as a percent increase of mouse survival time as compared to mice given PBS as control.

Brain Uptake Trials Involving p97-ADR

Mice received tail vein injections of ^{125}I-p97-ADR conjugates (SYN019, SYN020), ^{125}I-p97, ^{125}I-BSA, or ^{125}I-Lf (8 μl/g body mass, 3 μg protein/ml). One hour after injection, mice were anesthetized (ketamine-HCl 100 mg/kg, xylazine 10 mg/kg) and a 300 μL sample of blood removed by cardiac puncture. The left atrium was snipped with scissors and the mouse was perfused with heparinized saline from a peristaltic pump through a 27 gauge needle. After perfusion, the brain was removed and CPM per gram of tissue determined using a Gamma counter (CobraII, Packard, IL).

Statistical Analysis

Means are reported with their standard errors. Groups were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple or Tukey's range test. For regression analysis, the least squares method was used, as well as the difference between the slopes of regression lines.

Results

Study design

The biodistribution of intravenously injected p97 vector, free drug, or p97-drug conjugates were examined initially. The modulation of growth of representative classes of tumors growing in peripheral tissues outside the BBB (subcutaneously) and growing cloistered behind the BBB (intracranially) were examined including resident (rat C6) glioma and metastatic (human ZR-25-1) mammary tumor cells. Both tumor types present with reliable characteristic of growth, which parallels patterns witnessed in the equivalent diseases in humans. They both have 100% take rates in nude mice and reproducibility in survival patterns [66,67]. We chose to examine the therapeutic effects of ADR and p97-ADR conjugates rather than PTAX and p97-PTAX because C6 is resistant to the cytostatic activity of PTAX [68]. We recorded tumor size and mouse mortality in groups of nude mice inoculated either subcutaneously

or intracranially with aggressively growing xenogeneic tumor types (rat glioma and human mammary tumors) which were then treated with p97-ADR, free drug or saline controls.

Stability and Biodistribution of p97

The stability of iodinated p97 was verified at room temperature in buffer and in serum samples taken at different time points after injection. Plasma analysed by SDS-PAGE at 1 hour post-*i.v.* injection showed the great majority of ^{125}I remained at the p97 MW position (Figure 1a). Its appearance in urine was accompanied by a proteolytic cleavage event (Figure 1). One hour after injection, ^{125}I-p97 levels in the blood, kidney, bladder, liver, spleen, gallbladder, eye, heart and lung were all higher than in the central nervous system (Figure 2a). The initial uptake of p97 in the kidney, liver and spleen increased rapidly within minutes of injection and then decreased within the subsequent few hours. After 1 hr, p97 was found to accumulate in the brain while its level in all other organs decreased. The decrease in p97 concentration in plasma over time is shown in Figure 2b, whereas 10 minutes after injection of iodinated p97, it begins to accumulate in the brain (Figure 2c). After 6 h, the majority of ^{125}I-p97 associated with the brain was localized to the brain parenchyma (83%), and not the blood vessels (Figure 3). Unlike the other organs, the brain continued to accumulate p97 at 24 hours after injection. After 24 hrs, the total accumulation of p97 in the brain reached 1–2% of the injected dose.

Entry of p97 into the Brain via a Receptor-Mediated Process

Supporting the hypothesis that p97 enters the brain through a receptor-mediated process on the BBB, we have found that the rate of uptake into the brain of *i.v.* injected ^{125}I-p97 can be inhibited in a dose related manner by an excess of unlabeled p97 (Figure 4), supporting previously published data [52]. This "cold block" signifies competition for a receptor to the point of saturation. Competition with an excess of nonradiolabeled p97 in other organs such as the liver, kidneys and lungs is very low (data not shown), suggesting that p97 is not taken up in these organs in a receptor-mediated fashion. It also suggests that the brain expresses a higher concentration of receptors for p97 than these other organs. Using multiple-time regression analysis [57,69] we found that the influx constant (K_i) is higher for p97 (Figure 4) than that for albumin [70], which crosses the BBB through extracellular pathways, again suggesting the presence of a receptor mediated system of ^{125}I-p97 transport. The entry of ^{125}I-p97 into brain, measured by the brain/serum ratios of radioactivity (μl/g), was linear when plotted against exposure time. A dose-related self-inhibition of the K_i for ^{125}I-p97 brain uptake was achieved by co-administrating an excess of unlabeled p97 protein. The brain influx rate (K_i) for each group is as follows: (a) ^{125}I-p97 only: 2.02±0.28 ml/g-min; (b) ^{125}I-p97 plus 12 mg of p97: 0.45±0.08 ml/g-min; and (c) ^{125}I-p97 plus 15 mg of p97: 0.16±0.19 ml/g-min. The difference in the regression lines slopes was statistically significant [$F(2,17)=21.8$, $P<0.0001$]. The finding that the entry of p97 into the brain can be significantly inhibited by unlabelled p97 suggests that this may involve a receptor-mediated process. The addition of unlabeled p97 did not change the entry rate for ^{99m}Tc-albumin (overall $K_i=20.1\pm0.03$ ml/g-min).

Microscopic Visualization of p97-Conjugates in the Brain

Drug carriers that efficiently piggyback drugs into the brain after peripheral administration would be expected to first localize to the brain then rapidly translocate within the parenchyma, and then perhaps eventually emerge in the cerebrospinal fluid. Thus we examined if (i) fluorescence-labeled conjugates of p97 or (ii) unlabeled could efficiently traverse the BBB and appear in the parenchyma of the brain. One hour after *i.v.* injection, p97-Alexa 488 conjugate (Figure 5a) and p97 (Figure 5c) could be seen both in the lumen of brain microvasculature and within cortical cells by fluorescence microscopy. Fluorescence in cortical cells exhibited a punctuate distribution in the cytoplasm but not in the cellular processes. This staining pattern was in contrast to fluorescence associated with Tf-Alexa 488 (Figure 5b) and Tf (Figure 5d). Punctate cytoplasmic staining in cortical cells was not observed for Tf-Alexa 488 or Tf, despite strong staining in the microvasculature similar to that observed for p97. This is consistent with previous observations that p97 can effectively traverse the BBB. The integrity of the BBB following *i.v.* injection of p97-DIG conjugate (Figure 5e) was examined using EM. Specific staining for p97-DIG was observed in the brain parenchyma abluminal to the microvasculature. Despite weak fixation, the BBB was demonstrated to be intact, indicating that p97-DIG crossed into the brain without disruption of the BBB. Furthermore, p97-PTAX was clearly identified in brain parenchyma (Figure 5f), whereas free PTAX could not be seen in brain but was visualized in other organs such as heart and liver (not shown). These data indicate that p97 can transport a wide variety of molecular compounds across the BBB and that the drugs conjugates remain intact following delivery to the brain.

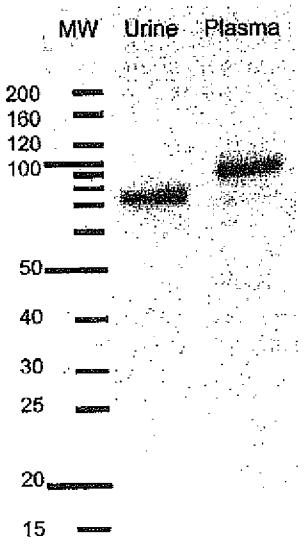

Figure 1. Stability of p97 in the mouse 1 hr. after intravenous injection. Iodinated p97 protein is found intact in plasma after 1 h post-*i.v.* injection, but appears to undergo a cleavage event prior to appearance in urine.
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Stability and Biodistribution of ADR and PTAX Conjugates

The stability of p97-PTAX conjugate was verified in buffer and in serum samples at ambient (room) temperature, taken at different time points after injection by SDS-PAGE analysis and HPLC analysis. As determined by HPLC analysis, the conjugate stored in buffer was stable for over 24 hrs at room temperature

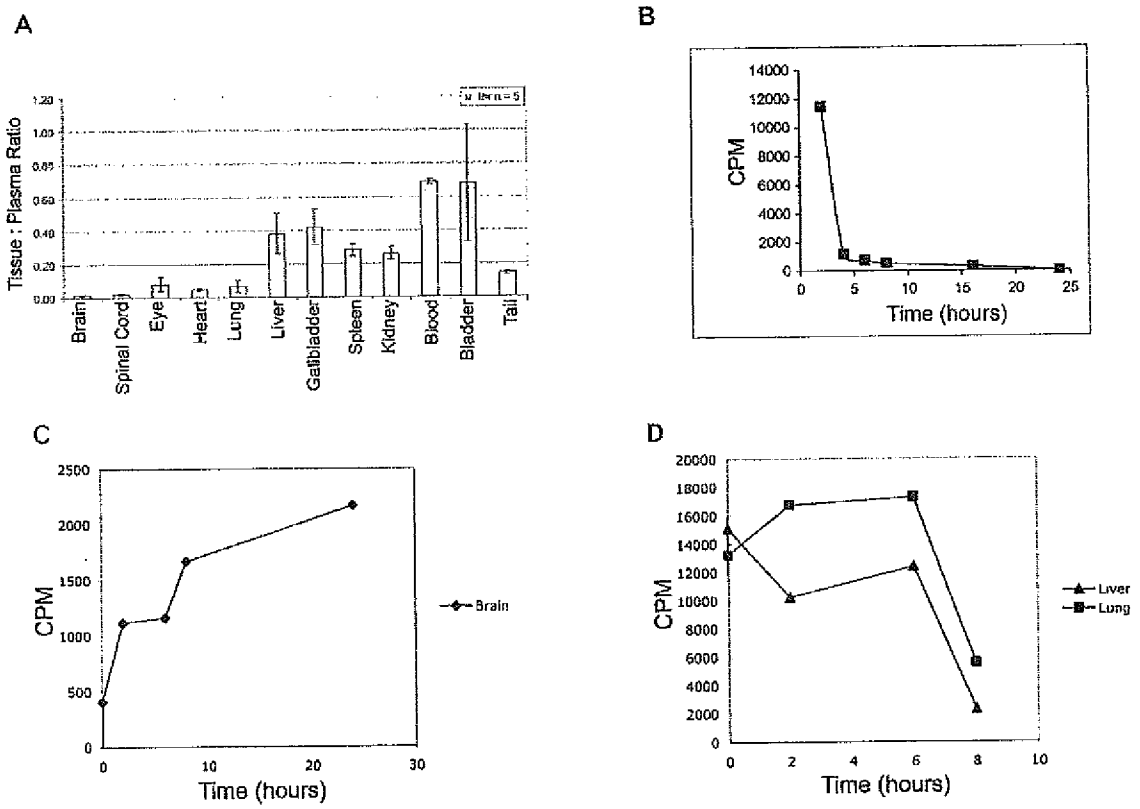

Figure 2. Distribution of p97 in tissues and organs after intravenous injection. **(a)** Tissue plasma ratios of iodinated p97, normalized by organ mass, in a variety of organs at one hour post-injection, including: whole brain, spinal cord, eye, heart, lung, liver, gall bladder, spleen, kidney, blood, bladder, and tail, $n=5$ mice. **(b)** The plasma concentration radioactivity, normalized by plasma mass, of ^{125}I-p97 after *i.v.* injection decreases over time. The figure represents data from one mouse. **(c)** The radioactivity, normalized by plasma mass concentration, of ^{125}I-p97 after *i.v.* injection increases in brain over time. The figure represents data from one mouse at each time point.
doi:10.1371/journal.pone.0002469.g002

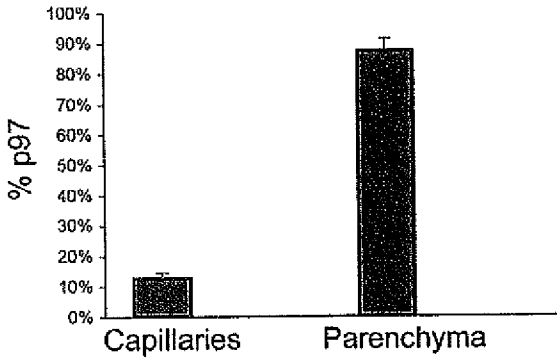

Figure 3. Intravenously injected p97 is able to cross the BBB and enter the brain. At 6 h post-injection greater than 80% of the p97 in the brain is found in the parenchyma rather than the capillary blood vessels.
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and in the blood for up to 4 hours (Table 1). The p97-PTAX conjugate (Figure 5f) and its metabolites were detected in brain tissue but free PTAX was not detected in the brain (Table 1).

The stability of the p97-ADR conjugates was directly assessed by analyzing tissues and body fluids post injection. p97-[^{14}C]ADR conjugate was stable in mouse serum for at least 1 h, with some degradation observed after 18 h (Figure 6a). Protein-associated radioactivity after TCA precipitation remained above 80% for the duration of the experiment. The MSR for the p97-ADR conjugates ranged between 5 and 6. The brain uptake of ^{125}I-p97 and ^{125}I-p97-ADR was compared to ^{125}I-BSA and ^{125}I-Lf one hour after a single bolus tail vein injection (Figure 6b). Uptake of ^{125}I-p97 and ^{125}I-p97-ADR conjugates (SYN019 and SYN020) into the brain were almost ten-fold higher than that of BSA or Lf. These data show that conjugation of ADR to p97 does not affect the transport of p97 into the brain.

Effect of p97-ADR Conjugate on Subcutaneous Tumors

Treatment of C6 gliomas grown subcutaneously in mice was performed with both p97-ADR conjugate (SYN002) and free ADR. Both significantly ($p<0.01$* ANOVA) inhibited tumor growth by over 50% during a 20 day period when compared to PBS treatment (Figure 7a). The ADR doses (4 mg/kg, total) used

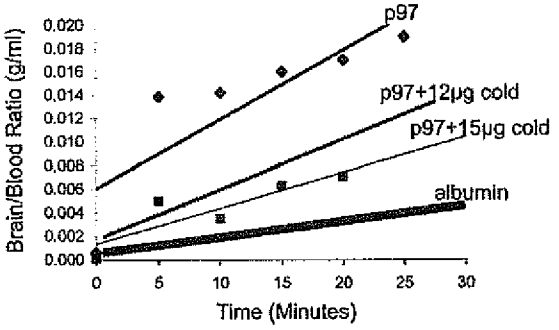

Figure 4. Multiple time-regression analysis of the rate of p97 entry into the brain. The entry of ^{125}I-p97 into the brain, measured by the brain/serum ratios of radioactivity (µl/g), was linear when plotted against exposure time. Addition of unlabeled p97 dose responsively inhibited the entry of the radiolabeled ^{125}I p97 protein. The influx rate (K_i) for each group was found to be as follows: (i) ^{125}I-p97 only: 2.02±0.28 ml/g-min; (ii) ^{125}I-p97 (plus 12 µg of cold p97): 0.45±0.08 ml/g-min; and (iii) ^{125}I-p97 (plus15 µg of cold p97): 0.16±0.19 ml/g-min. The difference among the slopes of the regression lines was statistically significant, $p<0.0001$. The graph represents one mouse.
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in this trial were lower than those typically used in chemotherapy (10–15 mg/kg). The SYN002 conjugate was slightly more effective than treatment with an equivalent amount of pure ADR.

Effect of p97-ADR Conjugation on Cardiotoxicity

There were significant differences in the serum levels of the two markers of cardiac damage, LDH and CPK after treatment of the animals (Figure 7b), as measured at the end of the trial (day 20). Treatment with ADR alone increased the level of both of these markers by over three-fold. Treatment with an equivalent amount of ADR conjugated to p97 (SYN002) had little effect on the level of these markers of cardiac cell damage, implying that conjugating ADR to p97 may reduce the cardiotoxic effects of ADR treatment. In all trials, the body weight of the mice did not appear to be affected by the treatments when compared to PBS controls.

Efficacy of p97-ADR Conjugates on Intracranial Tumors

The dosing schedules and different trials are outlined in Table 2, while the results of the treatments are presented in Table 3. In Trial 2 (Figure 8a) where the mice with intracranial ZR-75-1 mammary tumors were treated with p97-ADR SYN002 via tail vein injections, mean and median survival times were increased by 77% and 20.8% compared to the PBS treated group. It is interesting that treatment with free ADR alone appeared to reduced the mean and median survival of the mice suggesting greater global organ and tissue toxicity due to free ADR in these mice. It should also be noted that the total ADR injected in the form of conjugate was four times less than that of free ADR. A higher dosage of p97-ADR might further increase survival rates. Two mice in the SYN002 treated group survived to 50 days and were reported, after autopsy, to be tumor-free survivors. In Trial 3 (Figure 8b) where mice with intracranial C6 gliomas were treated with SYN018 via tail vein injections, mean and median survival times were increased by 40% and 44% respectively compared to the PBS treated group. The efficacy of the p97-ADR conjugates, SYN002 and SYN018, did not appear to be affected by differences in the preparation and both were effective in the treatment of tumors. Significant increases in survival time were again achieved

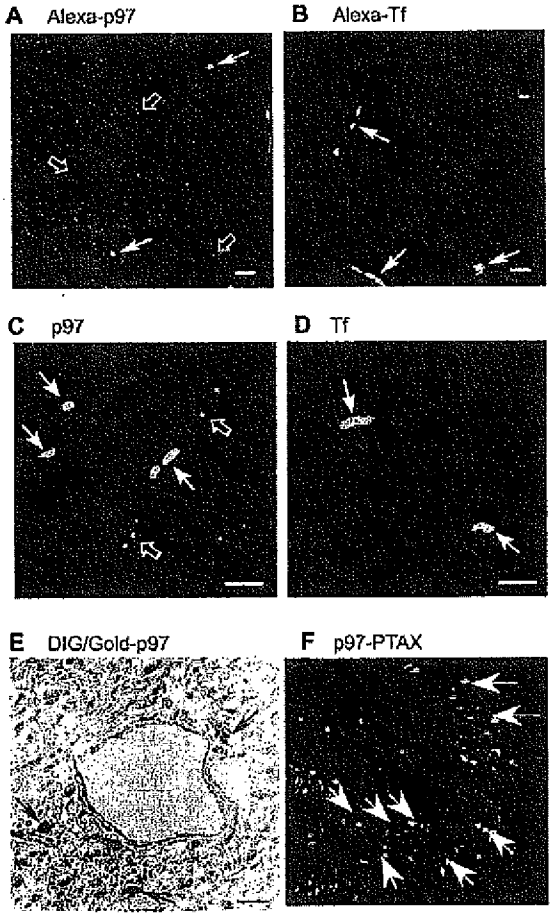

Figure 5. Visualization of p97 and Tf uptake in the brain. (a) Alexa 488 labeled holo-p97 and **(b)** Alexa 488-holo-Tf appear in the brain after one hr following injection in mice. Although p97 and Tf can be seen in the microvessels of respective mice (solid arrows), p97 appears to transcytose the BBB more efficiently than Tf and exhibits a punctate distribution in the cytoplasm of cerebral cortical cells (open arrows). Immunohistochemistry of **(c)** holo-p97 and **(d)** holo-Tf in the brain one hr after intravenous injection, using respective antibodies, show that although the two proteins are seen in the microvessels of respective mice (solid arrows), p97 appears to transcytose the BBB more efficiently than Tf and exhibits a punctuate distribution in the brain parenchyma (open arrows). Scale bar represents 5 µm. **(e)** After DIG-labeled p97 was injected into a mouse, the brain was harvested, sectioned, and the p97 localized with colloidal gold conjugated anti-DIG antibody and visualized by gold enhancement. Although parenchymal structures are weakly fixed, this EM shows that DIG conjugated p97 crosses the intact BBB and can be seen in the brain parenchyma. **(f)** Fluorescent PTAX is clearly shown within hipocampal brain sections of mice after the 5th injection (solid arrows).
doi:10.1371/journal.pone.0002469.g005

with relatively small total doses of ADR borne as p97-ADR. In Trial 4 where mice with intracranial C6 gliomas were treated with SYN002 via intrajugular vein injections, mean and median survival times were increased by 28% and 35% respectively when compared to the PBS treated group. In this trial, treatment with free ADR also improved the mean and median survival times of the mice but the improvement was considerably less than that of

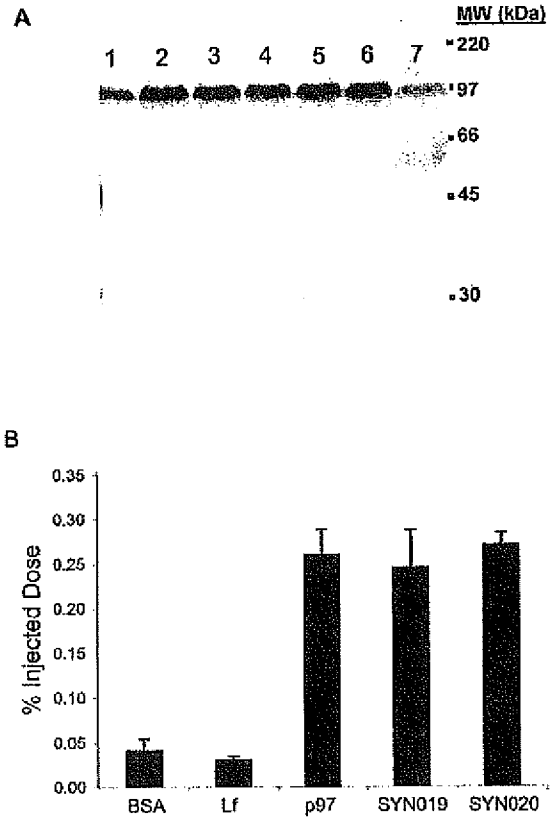

Figure 6. The stability and distribution of p97 and p97-conjugates in the brain of mice. **(a)** p97-ADR[^{14}C] appears to be stable in mouse serum over time. Lanes. 1 = 0 (minutes) and 90% (TCA precipitable counts), 2 = 10 min/92%, 3 = 20min/94.7%, 4 = 30min/90.5%, 5 = 40min/83.9%, 6 = 60min/93.9%, and 7 = 18 hours/83.3%. The position of the [^{14}C] molecular weight markers are as indicated. **(b)** A comparison of protein and protein conjugates crossing the BBB in mice 1 h after tail vein injection. CPM per g of brain tissue as a percentage of the total CPM injected. The albumin (BSA) (n = 2) and lactoferrin (Lf) (n = 2) show low levels while p97 (n = 5) and both p97-ADR conjugates SYN019 (n = 3) and SYN020 (n = 3) are significantly higher at one hour post injection.
doi:10.1371/journal.pone.0002469.g006

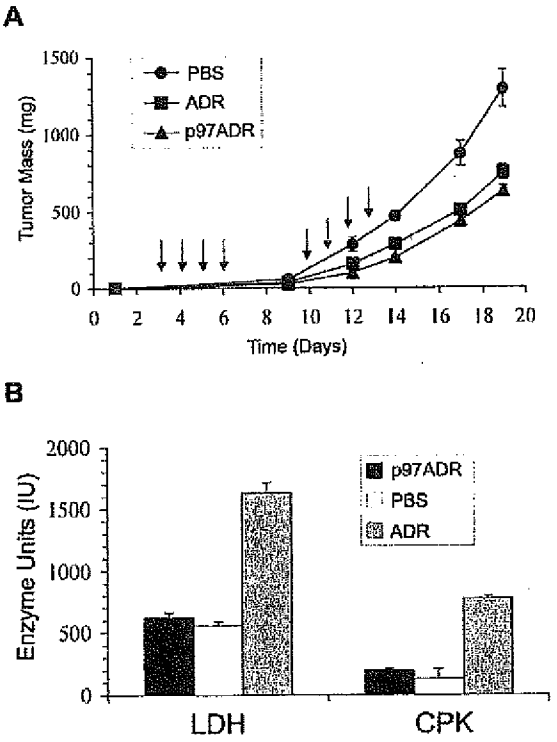

Figure 7. Mice bearing subcutaneous tumors were treated with p97-ADR conjugates. a) Tumor growth of subcutaneous C6 glioma mass in mice treated with p97-ADR conjugate SYN002 (4 mg/kg ADR, *o-o*), ADR (4 mg/kg, *v-v*) or PBS (λ-λ). Arrows indicate injection schedule (also refer to Trial 1 in Table 1). (n = 9 for each treatment group) b) Activity of LDH and CPK in serum from mice treated with the p97-ADR conjugate SYN002 (4 mg/kg ADR), ADR alone (4 mg/kg) or PBS. Values represent mean values±standard deviation (n = 3).
doi:10.1371/journal.pone.0002469.g007

the p97-ADR (see Table 3), and resulted in higher over toxicity to the mice. Compared to Trial 2, efficacy of the p97-ADR did not appear to be affected by injection method, either tail vein or intrajugular vein. Therefore, the three studies showed that treatment with the p97-ADR conjugates were able to significantly extend the survival of mice with intracranial C6 gliomas or ZR-75-1 mammary tumors.

Discussion

The present study describes the first protein based 'ferrying' system that can be used as a brain delivery vehicle for traversing therapeutically efficacious concentrations of drugs into the brain for the treatment of neurological disease.

We have demonstrated that recombinant human p97, injected into experimental mice, showed few signs of molecular breakdown in circulating blood, even after 8 hours. In time-course studies, we showed that 10 minutes after the injection of radio-iodinated p97, more radioactivity was detected in the brain than after injection of radio-labeled albumin control. One hour after injection, ^{125}I-p97 radioactivity, normalized per mg of tissue wet weight, in the blood, kidney, bladder, liver, spleen, gallbladder, eye, heart and lung were all higher than in the central nervous system. The initial uptake of p97 in the kidney, liver and spleen increased rapidly within minutes of injection. However, after 1 hr, p97 was found to accumulate in the brain while its uptake in all other organs fell to the same level as the albumin control. Unlike the other organs, the brain continued to accumulate p97, even 24 hours post injection. Over this 24 hr period, the ratio of counts per minute per mg due to ^{125}I-p97 in the brain compared to that in the plasma reached 10:1. After 24 hr, the total accumulation of p97 in the brain reached 1–2% of the injected dose equivalent to the ratio of brain to body weight-the first carrier system to approximate this biological feat. This is a much higher accumulation than that others have observed for chemotherapeutics to date [15,71,72]. For example, less than 0.1% of morphine accumulates in the brain over time[73]. The organ distribution of ^{125}I-p97, its brain accumulation over time and the fact that the BBB microvasculature lacks albumin receptors suggests that ^{125}I-p97 entry into the brain is likely due to a receptor. Accumulation of homologous (murine) p97 would possibly be even greater than of the

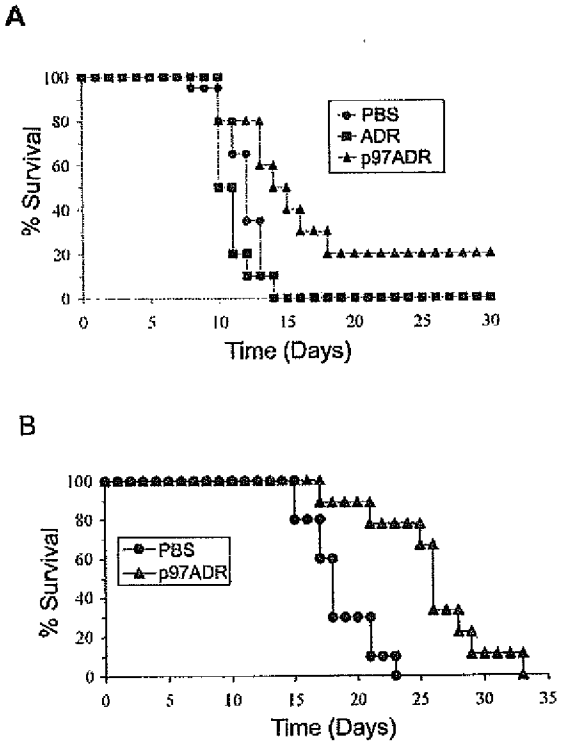

Figure 8. Mice bearing intracranial tumors were treated with p97-ADR conjugates. a) Percentage (%) survival of mice bearing intracranial ZR-75-1 mammary tumors and treated with p97-ADR conjugate SYN002 (5.5 mg/kg ADR, σ-σ), ADR (20 mg/kg, ν-ν) or PBS (λ-λ). b) Percentage (%) survival of mice bearing intracranial C6 glioma tumors and treated with p97-ADR conjugate SYN018 (0.49 mg/kg ADR, σ-σ) or PBS (λ-λ).
doi:10.1371/journal.pone.0002469.g008

heterologous (human) p97 studied here and perhaps bodes well for transfer of this technology into a clinical application in humans. In the present study, the transport of p97 across the BBB without altering BBB permeability was confirmed using microscopy (Figure 5). The uptake of Alexa-, DIG- or gold-conjugated-p97 and unconjugated p97 by the brain was observed using confocal microscopy and EM (Figure 5). Consistent with studies using radio-labeled compounds [39], the BBB appeared to be more effective in limiting the entry of Tf into the brain than p97. The distribution of Tf was limited to microvessels, whereas p97 was observed in the cerebral parenchyma (Figure 5).

To determine whether p97 completely traverses the BBB or still resides within the circulation (mainly capillaries), the compartmental distribution of ^{125}I-p97 after *i.v.* injection was analyzed by applying the capillary depletion method to the cerebral cortex. As the BBB isolates the cortex from all systemic influences, any systemically *i.v.* administered ^{125}I-p97 recovered from the brain parenchyma must have penetrated the BBB from the circulation. Six hours after *i.v.* injection into mice, we found most ^{125}I-p97 (over 80%) associated with the brain parenchyma rather than in the brain capillaries. The fluorescence distribution (Figure 5) makes it unlikely that p97 is attached to the luminal surface of the endothelial walls. These results indicate that ^{125}I-p97 does not associate with the CNS vasculature but is transcytosed through the endothelial cells into the brain parenchyma, consistent with past findings [10,44,74].

Supporting the hypothesis that p97 enters the brain through a receptor-mediated process on the BBB, we have found that the rate of uptake into the brain of *i.v.* injected ^{125}I-p97 can be inhibited in a dose related manner by an excess of unlabeled p97 [52]. This "cold block" signifies competition for a receptor, possibly a LRP receptor [52]. Using multiple-time regression analysis [57,69] we found that the influx constant is higher for p97 (Figure 4) than that for albumin, which crosses the BBB through extracellular pathways [70], again suggesting the presence of a receptor mediated system of ^{125}I-p97 transport, as suggested in previous reports [49,74]. In contrast, the addition of unlabeled p97 did not change the entry rate of ^{99m}Tc-albumin indicating that there was no disruption of the BBB due to injection of these radiolabeled proteins. We have also shown that PTAX-conjugated-p97 is transported to the brain (Figure 5f), whereas free PTAX could not be detected. We were able to identify PTAX and its metabolites in mouse brains after a cycle of 5 injections of p97-PTAX (Table 1).

Standard clinical chemotherapy for brain tumors includes highly lipophilic alkylating agents such as nitrosourea and temozolomide [72] which are able to cross the BBB. However, their effectiveness is limited by the low sensitivity of primary tumors to these drugs [75]. As a rigorous test of p97 as a drug delivery agent, we chose to use anti-cancer drugs with a high

Table 3. p97-conjugated ADR increased survival times in mice with intracranial tumors.

Trial	Compound	Mean Survival In Days	% Change in Mean Survival	% Change in Median Survival	Log Rank Significance
2 (Zr-75-1 Tumors)	PBS	10	-	-	-
	ADR	9.24	−7.6	−12.5	$p<0.05$
	SYN002	17.7	77	20.8	$p<0.005$
3 (C6 Tumors)	PBS	20.2	-	-	-
	SYN018	28.3	40	44	$p<0.001$
4 (C6 Tumors)	PBS	21.8	-	-	-
	ADR	24.13	10.7	13.6	$p<0.05$
	SYN002	27.9	28	35	$p<0.005$

The percent increase in survival times for mice with free or conjugated ADR compared to controls treated with PBS clearly indicates that ADR conjugation to p97 results in a survival advantage for mice with intracranial tumors.
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therapeutic index whose path to brain tissue from the circulation is normally blocked by the BBB. Thus we chose to study Paclitaxel (PTAX) and Doxorubicin (formerly Adriamycin (ADR)). PTAX was first identified in the bark of the Pacific yew tree, *Taxus brevifolia*, by Monroe E. Wall and Mansukh C. Wani [76]. Initially, there were concerns regarding potential environmental impact when it was learned that 1,200 kg of pacific yew tree bark yielded only 10 g of pure material [77]. In order to address this problem, Pierre Potier and then Robert A. Holton, completed the first semi-synthesis of PTAX from the needles of the English yew tree, *Taxus baccata* [77]. This was followed by the first total synthesis of PTAX by Holton and his colleagues [78,79]. Subsequently, fermentation techniques were developed to allow large scale production without invoking wide-scale decimation of Pacific or European Yew populations [80]. PTAX blocks cell division by binding and stabilizing microtubules, which comprise the cytoskeleton and the mitotic spindle [81]. It is used in the treatment of lung, ovarian, breast, head and neck cancers and advanced forms of Kaposi's sarcoma [77]. Unfortunately, PTAX has no beneficial clinical effect in halting the growth of brain tumors or extending the life of patients with brain tumors largely due to its inability to traverse the BBB [82,83]. We find that while p97-PTAX conjugates effectively cross the BBB, we chose to study the therapeutic effects of ADR because PTAX lacks tumoricidal or cytostatic activity against C6 gliomas [68].

Another common chemotherapeutic agent studied here, ADR, is an anthracycline glycoside. It was derived from a red-colored antibiotic produced from a strain of *Streptomyces peucetius* by a group at Farmitalia Research Laboratories working near the shores of the Adriatic Sea [84]. ADR is commonly used to treat leukemias, Hodgkin's lymphoma as well as solid tumors such as cancers of the breast, stomach, bladder, lung, ovaries, thyroid and soft tissue sarcomas, multiple myeloma, and others [85]. It has a number of possible mechanisms of action, including inhibition of DNA replication after it intercalates with DNA. ADR inhibits the progression of the enzyme topoisomerase II after it has broken the DNA chain to allow replication; it prevents the DNA double helix from being religated [86,87]. ADR is 500–3000 times more effective against glioma cells *in vitro* [88] than *in vivo*, but the presence of efflux pumps localized at the blood–brain barrier renders it ineffective against tumors in the CNS [12,89]. In addition, its effectiveness is limited by a short half-life *in vivo*, a large apparent volume of distribution that results in low brain tumor accumulation [90] and toxic side effects on normal organs, including the heart [91]. Therefore, in the chemotherapeutic trials conducted here, ADR conjugates were particularly good candidates for evaluation because free ADR is excluded from the brain by Pgp1 efflux activity and because ADR is completely ineffective in treating brain tumors [92] because of hindrance by the BBB. The p97-ADR conjugates were stable in serum (>18 hours) and two different batches had very similar properties. In all the trials, the total dose of conjugated ADR injected into mice was significantly lower than that of a typical therapeutic dose of free ADR (see Table 2). In Trial 1, we showed that p97-ADR (SYN002) was as effective in inhibiting subcutaneous glioma tumor growth as an equivalent amount of free ADR (over 50% reduction in tumor mass over a period of 19 days) (Figure 7a). The data further demonstrated that therapeutic amounts of ADR [30], could be delivered into the brain upon conjugation to p97. In Trial 5 (Table 2 and Figure 6b), we showed that similar amounts of p97 as p97-ADR conjugates (SYN019 and SYN020) were transported into the brains of mice after tail vein injection. Both were transported into the brain at significantly higher levels (approximately 10 fold higher) than lactoferrin control [48].

Free ADR has been shown to be cardiotoxic in some cases [64,65] and it can cause neuro-toxicity when administered to the brain via partial permeabilisation of the BBB [93], limiting the amount of ADR tolerated by human patients. In Trial 1, two enzyme markers of cardiotoxicity, LDH and CPK, were monitored. When free ADR was administered, there was a significant increase in both markers. In contrast, when p97-ADR was administered, the levels of both markers were similar to that seen after PBS injections, indicating that conjugation to p97 could reduce the toxic effects of ADR in future clinical settings. It has been noted by others [33] that ADR cardiotoxicity can be reduced by conjugation of ADR to the peptides D-penetratin and SynB1. In addition, delivery of ADR via liposomes has resulted in reduced cardiotoxic effects [92]. It is not clear, whether conjugation or delivery by liposomes reduces the cardiotoxic effects of ADR by altering biodistribution or limiting the entry of ADR into cardiac tissue.

In the intracranial trials (Trials 2–4), treatment at 1 to 3 day intervals with the p97-ADR conjugates resulted in significant prolongation of survival of mice with brain tumors when compared to treatment with ADR alone. In Trial 2, mice bearing ZR-75-1 mammary tumor cells were treated, starting the next day, with the p97-ADR conjugate, SYN002. Untreated, the average mouse survival time was only approximately 10 days. Treatment with the conjugate raised mean and median survivals to 77% and 20% respectively. Notably, two mice out of the 10 in the treatment group survived for over 50 days and were tumor-free at autopsy, by careful gross (non-histological) inspection. In trials 3 and 4, mice were injected intracranially with rat C6 glioma cells and treated with the p97-ADR conjugates SYN002 and SYN018 either via tail vein injection (Trial 3) or via intra-jugular injections (Trial 4). These brain tumors grew slower than ZR-75-1 tumors resulting in an average survival time of 21 days for untreated animals [67,94]. In both trials, significant increases in mean and median survival were achieved with p97-ADR (Table 3). In Trial 3, p97-ADR treatment allowed the mice to live more than 8 days longer than PBS control-treated animals, resulting in a 40% increase in mean survival and a 44% increase in median survival over PBS controls. In Trial 4, also found that p97-ADR treatment allowed the tumor-bearing mice to live 8 days longer than PBS controls and 4 days longer than mice treated with free ADR. This worked out to a 28% increase in mean survival (35% increase in medial survival) over PBS, and a 16% increase in mean survival over free ADR. In other studies (not shown), treatment with p97 alone had no effect on tumor suppression. The total ADR injected into mice over a given trial was significantly less than a typical human clinical therapeutic dose administered as a single bolus (20 mg/kg or 60 mg/m^2 versus 23.2 mg/kg or 70 mg/m^2) [95,96]. Therefore, future trials will test whether higher total ADR dosage, delivered as p97 conjugates, will further increase survival times. Nevertheless, these studies demonstrate that p97, once conjugated to drug compounds, can cross the blood brain barrier at a similar rate to its normal (unconjugated) physiological efficiency and that the molecule remains intact following transcytosis but its payload is fully tumoricidally active once placed inside the brain and behind the BBB.

The presence of the BBB, although protective in design, not surprisingly limits the effectiveness of therapeutic drugs directed at the treatment of diseases of the brain. The BBB effectively prevents most drugs from reaching the brain, whether or not they are intravenously injected. Situations where this becomes most evident are during therapeutic applications such as treatment of neurophysiologic disorders (including lysosomal storage diseases), brain cancers (neuroblastomas, gliomas), infections and inflammation, trauma, and delivery of constructs used for gene therapy.

Four main routes for molecules to enter the brain include the transcellular pathway (crossing through individual endothelial cells), the paracellular pathway (crossing between adjacent endothelial cells), intrathecal injection into the space surrounding the spinal cord or intracerebral injection (mechanical disruption of BBB by needle). Certain molecules may passively diffuse through a lipid membrane, employing the transcellular route. Increased lipid solubility, lack of polarizability, changes in hydrogen bonding ability and molecular size may make this route possible. Generally, the more lipid-soluble a molecule is, the more readily it moves from the aqueous environment of the blood across the nonpolar (lipid) environment of the endothelial cell membrane and enters the brain [97]. Counteracting influences that may slow diffusion include pH, temperature (unlikely to be of pharmacological importance in the mammalian brain) and retention in the blood due to protein binding [97]. Molar excess refraction, or the approximate measure of total volume, also appears plays a role in passive diffusion since diffusion coefficients are inversely related to molecular size, such that smaller compounds diffuse faster than larger compounds. Interestingly, permeability across the BBB appears to be inversely correlated to molecular size, where the addition of nonpolar groups to smaller molecules actually increases their ability to cross lipid barriers [97]. The disadvantage is that with this method, increased penetration across all membranes, not only the BBB, is increased non-specifically.

Another general route for transfer into tissues is the paracellular route, between capillary endothelial cells in this case. However, in the BBB, the paracellular route is blocked by tight junctions, preventing diffusion of even small molecules such as ions and water. Temporary deliberate disruption of this barrier to deliver drugs may be achieved by intravenous injections of hyperosmotic solutions (*i.e.* 2M mannitol) [98–100] or of biologically active agents such as bradykinin or angiotensins [97,101]. However, the permeabilisation effects are neither confined to the brain, so there is no drug tissue-selectivity; nor are they molecularly selective within the brain. Ionic/chemical imbalances ensue, and plasma proteins can not find their way into the brain extracellular fluid. Osmotic shock is therefore bound to be harmful due to the non-specificity of the proteins and other molecules which may also enter the brain extracellular fluid along with the drug of choice. Highly active efflux systems also limit the passive diffusion of molecules across the BBB. Three families of large glycosylated membrane proteins that act at the BBB include Pgp1, multi-drug resistant proteins (MRP1 and MRP5), and organic anion transporting polypeptides (OAT3 and OATP1) [97,102]. All exhibit broad substrate specificity. Some progress is being made in animal models to identify compounds that temporarily inhibit efflux pumps while momentarily allowing normally excluded therapeutic compounds to enter the brain[103], though the apparent draw back appears to be unforeseen dose related toxicity.

Currently, direct intra-cerebral delivery (infusion or implantation) is the only effective brain treatment to allow increased drug delivery [101,104] that was previously described. However, the volume that can be delivered is limited (as compared to intravenous injection), and the drug must still diffuse to the brain parenchyma from the site of deposit to be effective. A high risk of infection and high neurosurgical costs are also involved in this approach. Other procedures for brain delivery, including delivery of microcapsulated drugs [105–107], and the exploitation of specific membrane transporters for conjugated drugs are being investigated. Furthermore, it has been found that antibodies conjugated to drugs, can cross the BBB as a result of their interaction with specific receptors, which suggests that such conjugates may be of value in the delivery of systemic-borne therapeutic agents to the brain [31,107,108]. Issues related to immune hypersensitivity using this approach must ultimately be addressed, perhaps by "humanizing" the potential antibody carrier, in order to use these conjugates repeatedly. Clearly, non-invasive methods based on using endogenous BBB shuttling compounds for the introduction of therapeutic compounds across the BBB and into brain parenchyma should be developed as therapeutic intervention in many neuropathologies may only then be achieved.

Melanotransferrin (p97) is one such endogenous shuttling protein that has clear potential as BBB drug delivery vehicle. It appears to offer many advantages over existing delivery molecules or systems. First, p97 is a protein found at low levels (<10 ng/mL) [54] in the blood of most normal individuals. Alzheimer's patients appear to be the lone exception identified to date, where levels in the blood may be two or more fold higher [44,54,109]. Therefore, inhibition of injected p97-drug conjugates by endogenous material that could competitively occupy receptors at the BBB appears to be minimal. Secondly, since the p97 appears to traverse the BBB as part of its normal function, its use in delivering drugs is not likely to result in p97-associated toxicity, though this has yet to be proven in a clinical setting. Thirdly, exogenously-introduced p97 can be expected to localize in concert with the tissue distribution of the target receptor. Thus it appears to localize to brain microvasculature and subsequently concentrates in brain parenchyma [52]. Therapies for neurological diseases can thereby be preferentially targeted to the brain. Fourthly, because it is an autologous human protein, repeated treatments are unlikely to result in immune hypersensitivity or in elimination by neutralizing antibodies in clinical therapies. Finally, the transport of p97 and p97-conjugates does not appear to be adversely affected by the antiport activities of pumps such as Pgp-1.

This study demonstrates the unique potential of using p97, as a 'Trojan horse' to ferry normally excluded therapeutic compounds, through the battlements of the BBB, thereby allowing forbidden cargo to traverse from the blood and emerge in the brain. The utilization of p97 as a shuttling platform is thus a new paradigm for carrier-mediated transport into the brain. New avenues should now be open to explore the generalized use of p97 to transport therapeutic compounds into the brain for the treatment of a variety of chronic and acute CNS diseases.

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This study is dedicated by WAJ to the memory of Alan F. Williams.

Author Contributions

Conceived and designed the experiments: WJ DK GA MU RG TV GK. Performed the experiments: GA MU QC ST GK. Analyzed the data: WJ DK GA MU RG TV QC ST GK CP. Contributed reagents/materials/analysis tools: WJ. Wrote the paper: WJ DK GA MU TV CP.

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Exhibit F

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Identification of a Novel Route of Iron Transcytosis across the Mammalian Blood–Brain Barrier

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ABSTRACT

Objective: This study was undertaken to assess the role of p97 (also known as melanotransferrin) in the transfer of iron into the brain, because the passage of most large molecules is limited by the presence of the blood–brain barrier, including that of the serum iron transporter transferrin.

Methods: To study the function of the soluble form of p97, we followed the uptake of radioiodinated and ^{55}Fe loaded p97 and transferrin by the brain during a 24-hour period.

Results: We show that the soluble form of p97 has the ability to transcytose across the murine blood–brain barrier, and its transcytosis can be inhibited in a specific manner. We also provide evidence that p97 transports iron into the brain more efficiently than transferrin.

Conclusions: These data support the idea that p97 is an important iron transporter across the blood–brain barrier in normal physiology and possibly in neurodegenerative diseases, such as Alzheimer disease, in which iron homeostasis in the brain becomes disrupted.
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KEY WORDS: P97, melanotransferrin, transferrin, iron transport, brain, blood–brain barrier

INTRODUCTION

The blood–brain barrier (BBB) is formed by specialized capillary endothelial cells that act as a selective barrier between the peripheral blood and the brain tissues. This barrier limits the passive diffusion of many molecules, such as hormones and ions, which act to maintain the unique environment of the brain. Although the presence of specific transport systems within the endothelial cells must ensure that the brain receives all necessary compounds, the mechanisms of selective trafficking of macromolecules and peptides through the BBB are not well understood.

One essential element for cellular metabolism is iron. Free iron is cytotoxic, however, and its level is tightly regulated in normal physiology. Abnormally high levels of iron leading to free radical formation have been demonstrated in various neurodegenerative diseases, including Alzheimer disease (AD) (13,24) and cancer (32). Mechanisms that underlie iron uptake into the brain have remained a central mystery in brain physiology. Because transferrin (Tf) is abundant in the blood and the classically defined Tf and transferrin receptor (TfR) mediate iron uptake

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into most cells, it was assumed that iron uptake by the brain is also carried out by the same system. Several studies, however, have come to challenge the exclusive role of Tf in brain iron transport.

Since TfR was first localized on cerebral endothelia in adult rats and humans (12), its participation in iron transport across the BBB has not been questioned. Further studies have confirmed the localization of TfR on the BBB (18,28), and monoclonal antibodies against TfR were shown to cross the BBB (16,21). In contrast, the brain distribution of Tf, the main ligand for TfR, is surprisingly limited to oligodendrocytes and not to endothelia (4,19). Furthermore, studies have shown that the activity of Tf alone cannot account for the iron accumulated in the brain (20,27). As an explanation, researchers suggest that Tf releases iron in the cytosol of cerebral endothelia (7,25,26). The iron released in the cytoplasm of the endothelial cell, in turn, might then become bound to endogenously expressed brain Tf, whereas the serum Tf transported into endothelia is recycled out of the brain. This was demonstrated using a perfusion model, in which only a small fraction of total Tf injected into mice transcytosed into the brain, whereas much of it was retained in the endothelia as an intracellular pool (7). More importantly, other studies have shown that hypotransferrinaemic mice deficient in Tf have a higher level of iron uptake by the brain than normal mice (6,31), suggesting that the current description of the Tf/TfR model for brain iron uptake is at the very least incomplete.

The disparate conclusions drawn from these studies could be reconciled if other modes of iron transcytosis across the BBB exist. We focused on another iron-carrying protein, p97 (also known as melanotransferrin), because of its colocalization with the TfR on the BBB (28), and studied its potential role for iron delivery into the brain. p97 belongs to a family of iron-binding proteins that include serum Tf, lactoferrin, and ovotransferrin. Human p97 shares 39% sequence identity with human Tf (23) and has been shown to bind iron (1,14). Unlike other Tf family members, this molecule exists in two forms, a glycosyl-phosphatidylinositol (GPI)–linked cell surface form and a secreted soluble form generated by alternative splicing (8,11,17). The level of soluble p97 is elevated in the serum of patients with AD, suggesting that the protein might contribute to the pronounced accumulation of iron in the brains of patients with AD (14,15). To further understand the mechanisms of iron uptake by the brain, we examined the ability of soluble p97 to cross the BBB in vivo.

MATERIALS AND METHODS

Protein Preparation

Soluble human p97 was obtained by concentrating and purifying soluble p97 transfected baby hamster kidney cell supernatant as described (33). Soluble p97 was first dialyzed to remove any bound iron before the experiment. Iron was loaded to p97 and mouse Tf by adding 10 nM of soluble apo-p97 to 10 mM of iron nitrilotriacetate (FeNTA) in the presence of 250 mM sodium bicarbonate. All experiments used human holo-p97 and mouse holo-Tf with the exception of microscopic examination in which human holo-p97 and human holo-Tf were used to distinguish the injected proteins from endogenous mouse proteins. For iodination, human p97, mouse Tf (Sigma, Oakville, Ontario, Canada), and bovine serum albumin (BSA; Sigma) were labeled using the chloramine T method and purified with Sephadex G25 columns (Pharmacia, Baie d'Urfé, PQ, Canada). Specific activities of ^{125}I-p97 and ^{125}I-Tf were determined to be 6×10^{18} cpm/mole and 9×10^{18} cpm/mole, respectively. Trichloroacetic acid precipitability was approximately 95%. For loading ^{55}Fe to proteins, 30 μL of 5 mM $^{55}FeCl_3$ (NEN, Boston, MA) was added to 60 μL of 25 mM sodium citrate. The solution was neutralized with 24 μL of 1 M sodium bicarbonate and 2.5 mg of human p97 or mouse Tf was added for 1 hour at 37 °C. The ^{55}Fe-loaded proteins were dialyzed in PBS twice to remove unbound ^{55}Fe citrate. Specific activities of ^{55}Fe-p97 and ^{55}Fe-Tf were 7.4×10^{14} dpm/mole and 1.9×10^{15} dpm/mole, respectively.

Transcytosis of p97 and Tf Across the BBB

For the ^{125}I-protein uptake time course and dose response experiment, 5.5×10^{-14} moles/g body mass of ^{125}I-human p97 or ^{125}I -mouse Tf was injected into the tail vein of C57Bl/6 mice (Fig. 1). In the competition study, 100 times concentration of cold human p97 or mouse Tf were added with the 5×10^{-14} moles ^{125}I-p97/g body mass and injected into the tail vein. For the time course of iron uptake into the brain, 2.5×10^{-11} moles/g body mass of ^{55}Fe-p97 or 1×10^{-11} moles/g body mass of ^{55}Fe-Tf was injected into the tail vein (Fig. 4a-c). After 1, 3, 6, or 24 hours, the mice were anesthetized with ketamine and xylazine. After a blood sample was collected from the right atrium, the mice were perfused with PBS containing 0.1% BSA through the left ventricle of the heart. Tissues were then collected and weighed, and radioactivity was determined using ei-

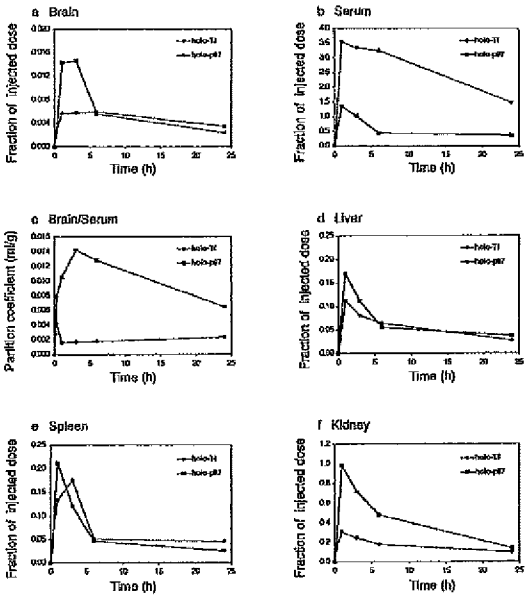

Figure 1. Uptake of p97 and Tf in vivo. The activity of ^{125}I-p97 (solid square) and ^{125}I-Tf (solid circle) in various tissues during a 24-hour period expressed as mass specific fraction of injected dose (g tissue/g body mass). (a) brain, (b) serum, (c) brain/serum partition coefficient (ml/g), (d) liver, (e) spleen, (f) kidney.

ther a gamma or scintillation counter. Blood samples were centrifuged at 11,000 rpm at 4 °C, the pellet was discarded, and the serum was collected for counting. The brain was capillary depleted as described by Triguero et al. (30). After dextran fractionation, light microscopy examination showed that the pellet consisted mostly of brain vasculature, whereas the top layer was essentially devoid of brain vasculature. In another set of experiments, 2.5×10^{-10} moles/g body mass of ^{55}Fe-p97 or 1×10^{-10} moles/g body mass of ^{55}Fe-Tf or 1.9×10^{5} dpm ^{55}Fe-citrate/g body mass was injected into the tail vein of mice (eight mice per chelate). One hour after injection, the mice were killed, and venous blood samples were taken for analysis. After perfusion, the brain was removed and processed whole for the measurement of radioactivity by liquid scintillation counting. The data were analyzed by ANOVA. All data are expressed as mass specific fraction of injected dose ([activity/g tissue]/[activity of injected dose/g body mass]).

Protein Analysis

To demonstrate that some iodinated holo-p97 crossing into the brain in the transcytosis studies remained intact, the mice were perfused with PBS 1 hour after 6×10^{6} cpm of ^{125}I-p97 was injected. Capillary depleted brain parenchyma was lysed in 1% Triton X-100 in 20 mM Tris-Cl, 2 mM EDTA, and 150 mM NaCl (pH 7.5) for 30 minutes at 4 °C. The lysate was collected and centrifuged at 11,000 rpm at 4 °C for 10 minutes. Because the level of radioactivity in the parenchyma after capillary depletion was too low to be detected using autoradiography, an immunoprecipitation using the monoclonal anti-p97 antibody L235 (ATCC, Manassas, VA) and Western blot was carried out for these samples as previously described (8).

RESULTS

Uptake of ^{125}I-p97 and ^{125}I-Tf by the Brain

Time course studies show that the initial amount of ^{125}I-p97 taken up by the brain rises sharply, reaching a peak of approximately 0.015 of injected dose within 1 hour of injection (Fig. 1a). Most ^{125}I-p97 is cleared from the brain within 5 hours, and the level returns to baseline in 24 hours. In comparison, the uptake of ^{125}I-Tf by the brain reaches a smaller plateau (0.005 of injected dose) 1 hour after injection and is gradually cleared during 24 hours. This is in sharp contrast to the profile in serum in which ^{125}I-Tf reaches a peak that is 2.6-fold greater than the ^{125}I-p97 peak. Furthermore, ^{125}I-p97 in the serum is cleared within 5 hours, whereas ^{125}I-Tf remains in the serum for up to 24 hours after injection. The brain to serum partition coefficient (mL/g) for ^{125}I-p97 is sevenfold higher than for ^{25}I-Tf, indicating preferential uptake of p97 by the brain (Fig. 1c). In contrast to the uptake by the brain, ^{125}I-p97 and ^{125}I-Tf are quickly incorporated into the liver and spleen reaching comparative peaks and are cleared within 5 hours of injection (Fig. 1d and e). A different pattern was noted for kidney, in which ^{125}I-p97 uptake was approximately threefold greater than ^{125}I-Tf (Fig. 1f).

To show that p97 that crosses the BBB into the brain remains intact, mice were injected with p97, and proteins were recovered from the parenchyma after perfusion and capillary depletion (30). Immunoprecipitation of the mouse parenchyma demonstrate a band at 97 kDa, suggesting that a significant portion of the injected p97 remained intact 1 hour after injection (Fig. 2).

The ability of unlabeled p97 and/or Tf to inhibit ^{125}I-p97 uptake into the brain was examined to establish the specificity of this process. The data in Fig. 3 show that the uptake of ^{125}I-holo-p97 is

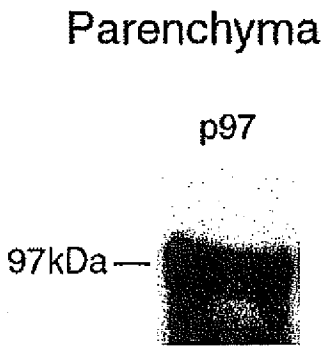

Figure 2. Recovery of p97 from the brain. Immunoprecipitation of p97 from parenchyma harvested 1 hour after injection, detected by chemiluminescence on Western blot.

partly inhibited by excess cold p97, whereas p97 uptake is less affected by the equivalent dose of cold Tf. The addition of cold p97 rapidly inhibits ^{125}I-p97 uptake and seems to cause its clearance from the system. Introduction of cold Tf, on the other hand, inhibits the uptake of ^{125}I-p97 in a less-specific manner than cold p97 and further reduces the clearance rate from the brain.

Uptake of ^{55}Fe-loaded p97 and ^{55}Fe-loaded Tf by the Brain

Efficiency of p97 and Tf to iron transport through the BBB into the mouse brain was examined by injecting ^{55}Fe-loaded p97 or Tf in vivo (Fig. 4). After 1 hour, iron bound to p97 accumulated in the brain parenchyma and vasculature 6 and 10 times more than iron bound to transferrin, respectively. The results also show that p97 in the parenchymal fraction are higher than that of the endothelial fraction. The brain-to-serum partition coefficient for ^{55}Fe-p97 is eight to nine times higher than ^{55}Fe-Tf 1 to 6 hours after injection (Fig. 4c, d). Interestingly, after the initial peak, only a slight uptake of ^{55}Fe is observed in the brain by either p97 or Tf.

DISCUSSION

We have used a combination of physiologic and cellular techniques to experimentally address the hypothesis that the p97 molecule is capable of crossing the BBB and that it is a major mediator shuttling iron into the brain. The ability of p97 to transcytose the BBB in vivo was examined first (Fig. 1). Our data show that p97 can cross the BBB in its intact form within 1 hour of injection and is largely catabolized and eliminated from brain tissues within 5 hours (Figs. 1 and 2). Soluble p97 transcytosed through the BBB into the brain more readily than Tf, even though the level of Tf in serum remained consistently higher than p97. The level of p97 uptake was higher than Tf in the kidney, whereas in the liver and spleen, comparative uptake profiles of p97 and Tf were recorded. Taken together, these results indicate that a large fraction of Tf is not likely to be taken up by the brain under normal conditions. This is consistent with several studies indicating that serum Tf is not the exclusive carrier of iron into the brain (7,20,27). It should be noted that in this study we used the available soluble human p97 compared with mouse Tf. It is likely that the murine homologue of p97, when it becomes available, should perform as well or better than the human orthologue.

In addition, we show that cold p97 seems to inhibit ^{125}I-p97 uptake with higher specificity than cold Tf using an in vivo competition assay (Fig. 3). Collectively, these results suggest p97 could interact with a receptor or receptors in vivo. Recently, low-density lipoprotein receptor has been suggested as a possible receptor for p97 (5). Low-density lipoprotein receptor, however, interacts with a large number of ligands, and it is possible that other receptors exist, which are more specific for p97 binding. Further study is needed to link ligand-receptor interaction to receptor localization on cerebral endothelial cells.

There is growing interest in designing molecules that can be transported into the brain by means of specific receptors naturally expressed in brain capillary

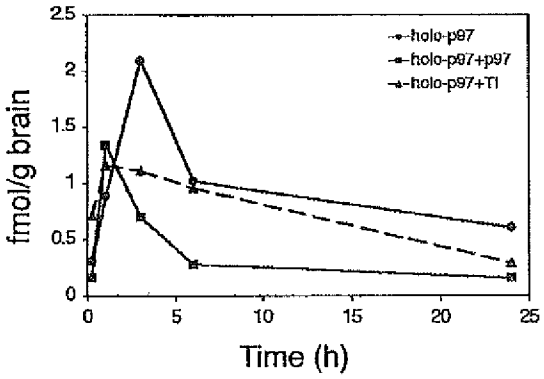

Figure 3. Uptake of ^{125}I-p97 is inhibited by p97 or Tf. The uptake of ^{125}I-p97 by brain tissue (circle) is inhibited more by the addition of 100× more cold p97 (square) than by the addition of an equal amount of cold Tf (triangle) during a 24-hour period.

endothelium. Some of the candidates for drug targeting to the brain include antibodies against TfR (16,21), glucose transporter (9,10), and a HIV gp120–derived peptide (2,3). The use of these molecules and others as carriers across the BBB might allow less-invasive and more-efficient delivery of therapeutic agents to the brain for the treatment of various neurodegenerative diseases and tumors. Because p97 seems to cross the BBB, it might act as an efficient carrier of therapeutic compounds and genes into the brain. Because p97 is a natural protein in human, it is unlikely to stimulate an immune response. Furthermore, the relatively rapid turnover rate of p97 in vivo compared with some antibodies suggests that p97 might be used for repetitive injection of conjugates.

After showing that p97 crosses the BBB from blood into brain parenchyma in vivo, we directly wanted to address whether p97 could mediate iron transport to the brain. The ability of p97 to bind iron has been established previously (1) and is also visible on a urea gel similar to Tf. The uptake of ^{55}Fe by the brain mediated by p97 was approximately eight to nine times higher than that of Tf after 1 to 6 hours after injection (Fig. 4).

Elevated levels of free iron are noted in various neurodegenerative diseases, including AD (24). It is well

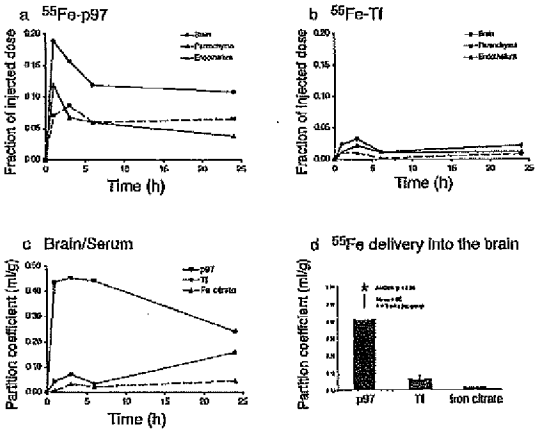

Figure 4. Iron transport into the brain. (a) ^{55}Fe transport into the entire brain (circle), brain parenchyma (square), and brain endovasculature (triangle) mediated by (a) p97or (b) Tf. The brain/serum partition coefficient (c) for ^{55}Fe transport is compared between p97 (square), Tf (circle), and Fe-citrate (triangle) during a 24-hour period. Statistical analysis (ANOVA) of ^{55}Fe transport into the brain 1 hour after injection of equivalent amounts of ^{55}Fe-p97, ^{55}Fe-Tf, or ^{55}Fe-citrate (d).

noted that the formation of free radicals resulting from excessive accumulation of free iron is particularly damaging to the sensitive tissues of the central nervous system (29). In addition to AD, excessive iron deposition and oxidative stress is associated with the pathologies of a number of neurodegenerative disorders, including aceruloplasminemia (34) and progressive supranuclear palsy (22). In addition to its potential usefulness as a drug shuttling molecule, p97 could play a central role in modulating iron transport in AD patients, because tantalizing studies have shown that the levels of p97 are elevated in the serum (14,15) and cerebrospinal fluid (14). Thus, the identity of the protein carrier that shuttles iron into the brain might be at last resolved, revealing a function for the secreted form of p97. Further investigations are being carried out to identify potential receptor(s), modes of transcytosis, and destinations of p97.

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